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Amelioration of 4′-Epidoxorubicin-induced Cardiotoxicity by Sodium Cromoglycate

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Abstract—*Epirubicin induces an important noncytotoxic release of histamine from rat peritoneal cells* in vitro. *This exocytotic response is inhibited by sodium cromoglycate, similarly to that elicited by the classic mast cell secretagogue, compound* 48/80.

Mast cells obtained from the peritoneal cavities of rats treated with epirubicin in vivo *were extensively degranulated; in contrast, samples obtained from rats pretreated with sodium cromoglycate showed normal appearing mast cells.*

When injected i.p., immediately before the antineoplastic agent, cromolyn significantly improved the survival time and the microscopic appearance of myocardial tissues of epirubicin-treated mice.

The results indicate that histamine release could play an important role in the pathogenesis of anthracycline-induced cardiotoxicity.

INTRODUCTION

4′-EPIDOXORUBICIN (epirubicin), the 4′-epimer of doxorubicin, was synthesized in an effort to find agents with equal or enhanced therapeutic efficacy but with reduced cardiotoxicity in comparison with the parent compound [1]. In clinical trials, the therapeutic effect was similar to that achieved with doxorubicin; moreover, at doses producing an equivalent antitumor effect, epirubicin induced less acute toxicity such as nausea, vomiting and myelosuppression than doxorubicin [1].

In different experimental animal models, epirubicin has been shown to be less cardiotoxic than the parent compound, and endomyocardial biopsies obtained from patients receiving epirubicin revealed that the tissue damage is qualitatively similar to that of doxorubicin at identical doses but quantitatively lower [2]. Nevertheless, the problem of anthracycline-induced cardiotoxicity has not thus far been resolved.

In previous studies conducted in this laboratory it has been shown that sodium cromoglycate, a mast cell stabilizer, significantly reduced the cardiotoxicity induced by an acute or chronic treatment with doxorubicin. The protective effect of this substance was most probably related to its inhibiting effect on doxorubicin-induced histamine release [3]. In fact, recent observations indicate that release of histamine and other vasoactive substances may be crucial in producing acute, subacute and chronic cardiotoxicity [4–7].

In this study, the effect of a pretreatment with sodium cromoglycate on epirubicin-induced histamine release *in vitro* and *in vivo*, and on the occurrence of the cardiotoxicity is examined.

MATERIALS AND METHODS

In vitro *studies*

Mixed peritoneal cells were obtained from 200–400 g male Sprague–Dawley rats (Charles River, Italy) by lavage of the peritoneal cavities with saline solution at 37°C. The physiological solution had the following composition: 1.54×10^{-1} M NaCl, 2.7×10^{-3} M KCl, 9×10^{-4} M $CaCl_2$, 5.6×10^{-3} M D-glucose, human serum albumin 1 g/l and 10% by volume of a Sörensen buffer containing 3×10^{-2} M $Na_2HPO_4 \times 7\ H_2O$ and 3.5×10^{-2} M $NaH_2PO_4 \times H_2O$. The pH of the solution was adjusted to 7.4 with 1 N NaOH before use.

The cells were sedimented by centrifugation at 200–250 ***g*** for 10 min, the supernatant fraction was removed and cells were resuspended in buffered

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medium. A pooled suspension from several rats was employed for an experiment (final cell suspension 2 ml solution per rat). The cell suspension contained approx. 10% mast cells and was used without further purification because only the mast cells in such a suspension contained histamine [8].

Two hundred microliters of a doubly concentrated solution of sodium cromoglycate (final concentration 10, 5, 2.5, 1.25, 0.62, 0.31, 0.15, 0.07, 0.03 mM) in physiological saline were added to 200 μl of the cell suspension and incubated at 37°C in a metabolic shaker. Where necessary, a solution (10 μl) of epirubicin (final concentration 100 μg/ml) was simultaneously added and the incubation continued for 10 min.

Samples were incubated in quadruplicate. Cells were separated from supernatants by centrifugation at about 200 ***g*** for 3 min. The cell pellets were resuspended in 400 μl of the saline solution and allowed to stand in a boiling water bath for 10 min to release residual histamine; the supernatants of controls were processed similarly. All the samples were assayed for histamine by the fluorimetric method of Shore *et al.* [9], omitting the extraction step. The amount of histamine released was calculated as a percentage of the total histamine present in the control suspensions. All values were corrected for the spontaneous release (approx. 5%) occurring in the absence of the inducer.

In vivo *studies*

Ten milliliters of Tyrode's solution alone or containing either epirubicin 0.5 mg/ml, epirubicin 0.5 mg/ml plus cromolyn 5 mg/ml, or cromolyn alone 5 mg/ml were injected into groups of three rats. The animals were sacrificed after 4 min and mixed peritoneal cells were obtained by injecting 20 ml of Tyrode's solution containing 0.1% human albumin i.p. The abdomens of the rats were gently massaged for 1 min and then drained. The peritoneal fluid containing mast cells was transferred to an ice cold tube and centrifuged at 250 ***g*** for 5 min at 4°C. The supernatant was discarded and the pellet was resuspended in 10 ml of Tyrode's solution and then recentrifuged (250 ***g***, 4°C). The supernatant was then removed and replaced by 5 ml of 2.5% glutaraldehyde in cold 0.1 M phosphate buffer (pH 7.4) for 45 min at 4°C, dehydrated with graded series of ethanol and embedded in Epon 812. Sections 1 μm thick were stained with 1% toluidine blue and observed by light microscopy. For electron microscopic examination, after fixation, the cell pellet was washed in buffer and postfixed in 1% osmium tetroxide in phosphate buffer, dehydrated in ethanol and embedded in Epon 812. Ultrathin sections for electron microscopy were examined after staining with uranyl acetate and lead citrate.

CD1 male mice (Charles River, Italy) with an average weight of 28–30 ***g***, were used. Animals were divided in six groups of 20 animals: group 1 received 20 mg/kg of epirubicin alone i.p.; group 2 received epirubicin as in group 1 plus 200 mg/kg of sodium cromoglycate i.p. immediately before epirubicin; group 3 received 8 mg/kg of epirubicin on days 1, 8 and 15 i.p.; group 4 received epirubicin as in group 3 plus 200 mg/kg of sodium cromoglycate i.p. immediately prior to each epirubicin injection. An additional group of 10 animals received cromolyn (group 5) i.p. on days 1, 8 and 15 without following epirubicin treatment; group 6 received i.p. injections of normal saline and served as controls. Animals were inspected daily for survival and general toxicity.

Five additional animals per group were sacrificed by cervical dislocation after 7 (groups 1–2) or 30 (groups 3–6) days. An autopsy was performed and specimens of the heart were collected and fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer at pH 7.4 and embedded in Epon 812. Semithin sections were cut at 1 μm, stained with 1% toluidine blue and observed by light microscopy. Material so prepared was examined and lesions were quantitifed by means of a Zeiss MOP Videoplan image analyzer.

Chemicals

Epirubicin was obtained from Farmitalia Carlo Erba, Milan. Disodium cromoglycate, histamine dihydrochloride and *o*-phthaldialdehyde were purchased from Sigma Chemical Co., St. Louis, MO. All other chemicals were of analytical grade.

RESULTS

In vitro *studies*

Figure 1 shows that epirubicin (100 μg/ml) induces a significant histamine release from rat peritoneal mast cells. A concentration of 100 μg/ml of epirubicin was used because this dose produced

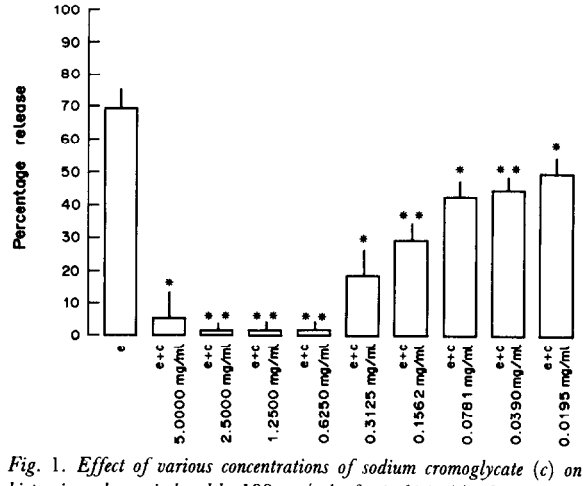

Fig. 1. Effect of various concentrations of sodium cromoglycate (c) on histamine releases induced by 100 μg/ml *of epirubicin (e). Columns are the means of four experiments and vertical bars are S.E. Significantly different from epirubicin alone, Student's* t *test for independent samples* (*$P < 0.01$, **$P < 0.001$).

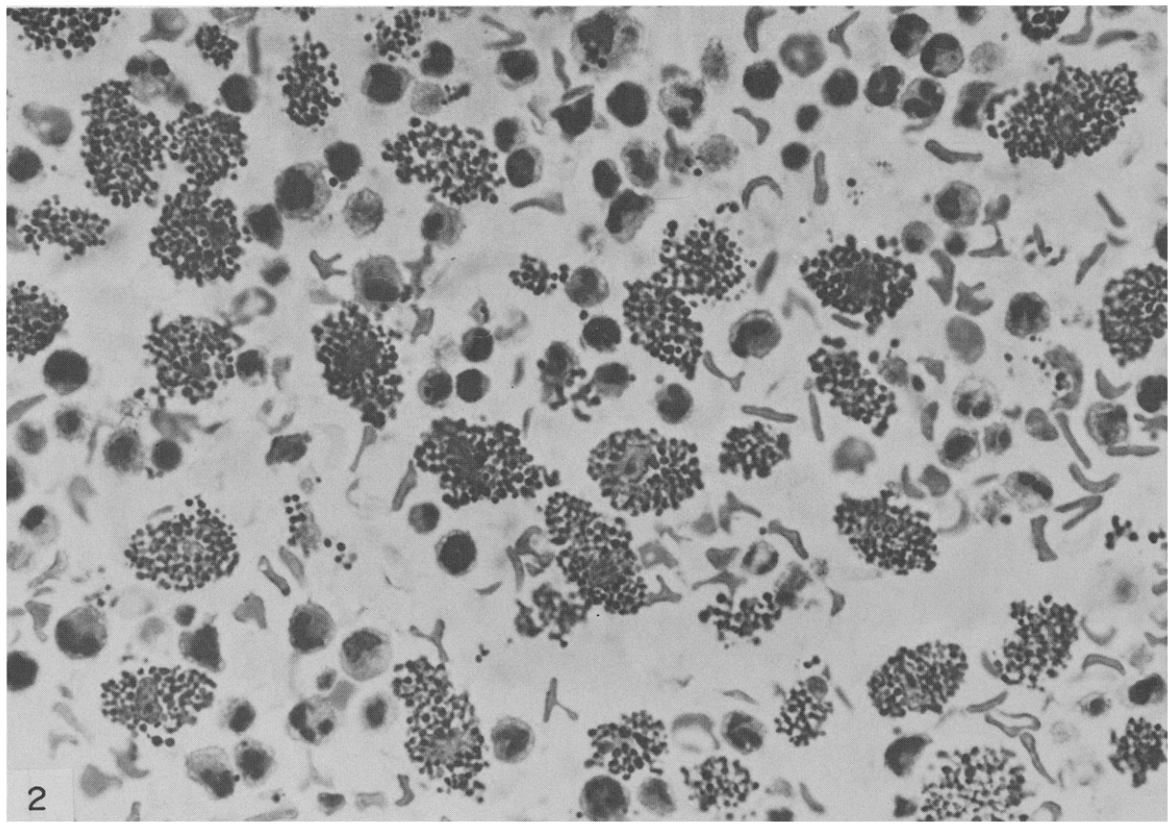

Fig. 2. Marked degranulation of peritoneal mast cells from a rat after i.p. treatment with epirubicin. Toluidine blue, × 630.

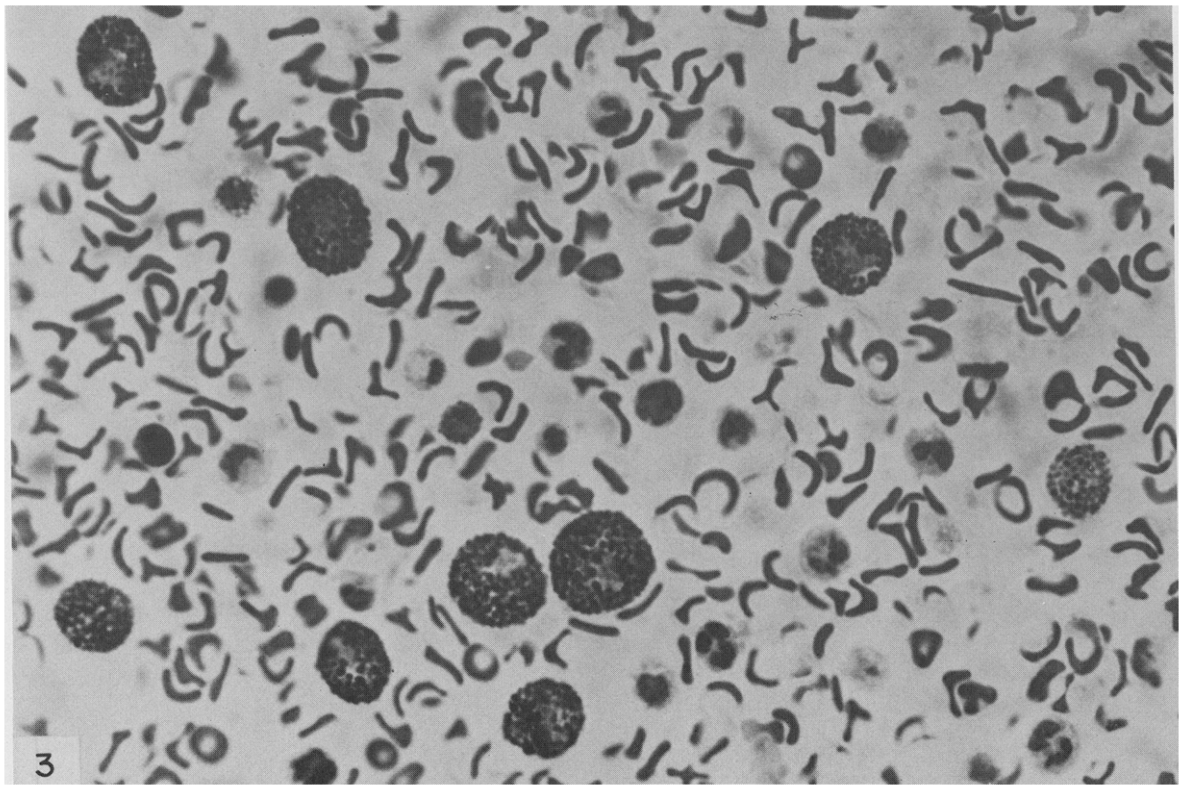

Fig. 3. No sign of degranulation in peritoneal mast cells from a rat after i.p. treatment with epirubicin plus sodium cromoglycate. Toluidine blue, × 630.

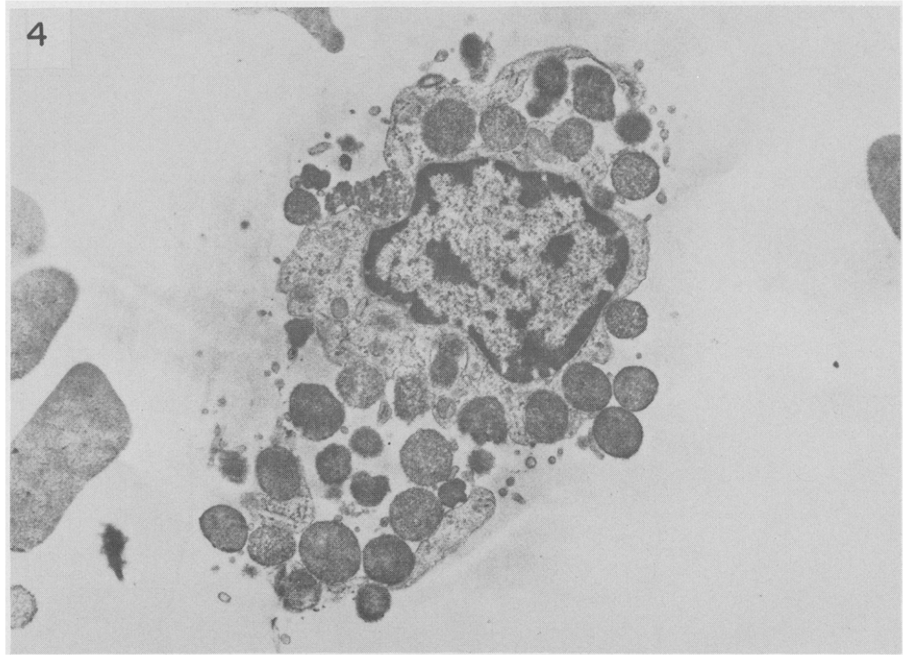

Fig. 4. Electron micrographs of mast cells from rat peritoneal cavity after exposure to epirubicin, showing severe degree of degranulation, × 4000.

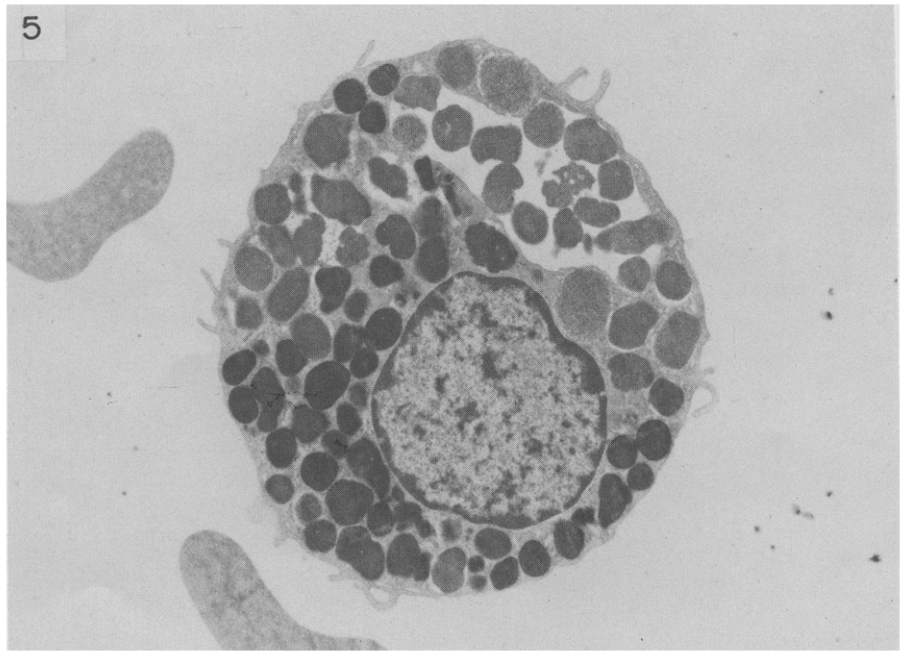

Fig. 5. Electron micrographs of mast cells from rat peritoneal cavity after exposure to epirubicin plus sodium cromoglycate, showing no sign of degranulation, × 4000.

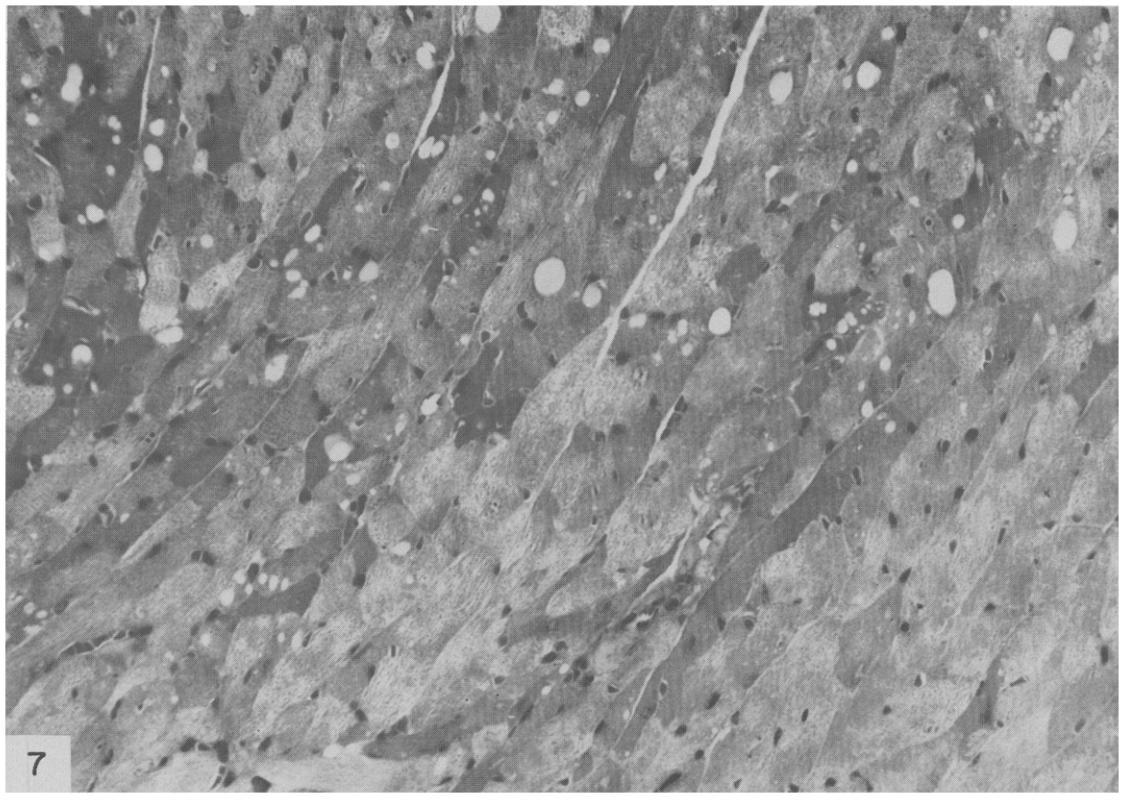

Fig. 7. Severe damage with myocytic vacuolization in the ventricular tissue of a mouse i.p. treated with epirubicin 8 mg/kg/*week* × 3 *weeks and sacrificed* 30 *days after the first injection. Toluidine blue.* × 250.

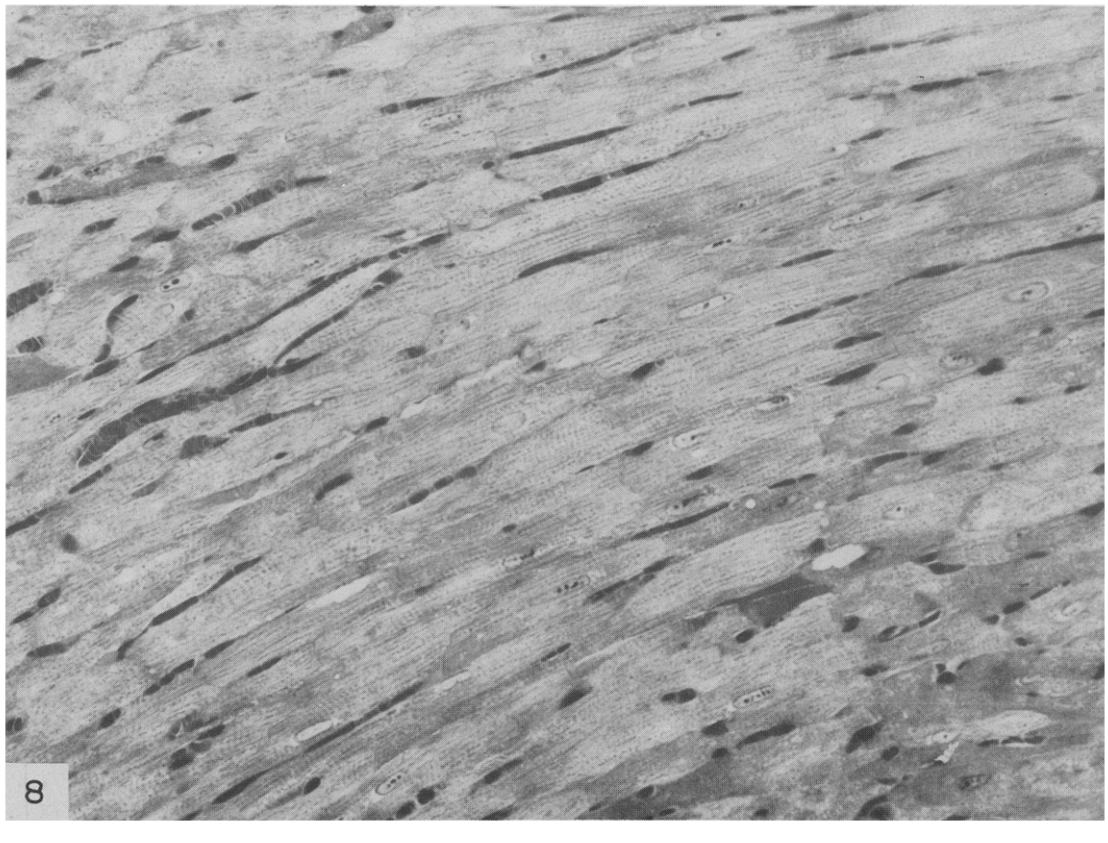

Fig. 8. Normal appearance of the ventricular tissue of a mouse i.p. treated with epirubicin 8 mg/kg/*week* × 3 *weeks* + *sodium cromoglycate* 200 mg/kg/*week* × 3 *weeks. Toluidine blue.* × 250.

the most important histamine release without cell disruption [10]. Histamine release was significantly inhibited by various doses of cromolyn (Fig. 1).

In vivo *studies*

When injected i.p. into rats, epirubicin induced a widespread degranulation of mast cells. This was shown by the optical microscopic observation (Fig. 2). The degranulation was minimal or completely absent in mast cells obtained from animals pretreated with cromolyn (Fig. 3). The ultrastructural features of mast cells of rats treated with epirubicin alone or epirubicin plus cromolyn are shown in Figs 4 and 5, respectively.

Epirubicin, when administered intraperitoneally acutely (20 mg/kg) or chronically (8 mg/kg/week × 3 weeks) to CD1 mice, caused high mortality. Pretreatment with cromolyn significantly improved the survival time of animals (Fig. 6). The epirubicin-induced cardiac lesions observed in this study were similar to those previously described in other animal studies (Fig. 7). These lesions were significantly reduced in mice pretreated with cromolyn (Fig. 8). Table 1 shows data on the vacuolization obtained by means of the image analyzer.

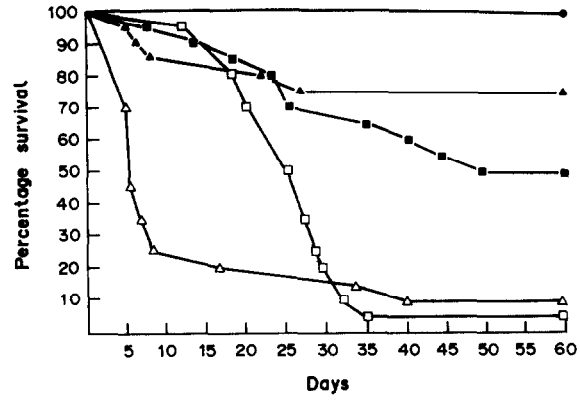

Fig. 6. Cumulative mortality data for mice receiving epirubicin 20 mg/kg *i.p.* (△), *epirubicin* 20 mg/kg *i.p.* + *sodium cromoglycate* 200 mg/kg *i.p.* (▲), *epirubicin* 8 mg/kg/*week* × 3 *weeks i.p.* (□), *epirubicin* 8 mg/kg/*week* × 3 *weeks i.p.* + *sodium cromoglycate* 200 mg/kg/*week* × 3 *weeks i.p.* (■) *or sodium cromoglycate* 200 mg/kg/*week* × 3 *weeks i.p.* (●).

DISCUSSION

This paper describes the effect of disodium cromoglycate on epirubicin-induced histamine release *in vitro* and *in vivo*. Epirubicin, like adriamycin, elicits a true exocytotic response from rat peritoneal cells, independent from its cytotoxic action, and resembles that induced by the classic mast cell secretagogue compound 48/80 [10]. Disodium cromoglycate, already used in limiting histamine release produced by compound 48/80 and other secretagogues [11], was extremely active also in inhibiting epirubicin-induced histamine release from rat peritoneal cells *in vitro*.

Light and electron microscopic examination of peritoneal mast cells obtained from rats treated with epirubicin i.p. revealed that histamine release was associated with an extensive degranulation of mast cells; however, complete destruction of the mast cells was not observed. The morphologic findings were similar to those observed after exposure of mast cells to adriamycin [2] or other agents which cause degranulation of mast cells such as compound 48/80 [11].

The search for congeners of doxorubicin with equal or enhanced therapeutic efficacy, but with reduced cardiotoxicity, led to the synthesis of a number of analogs including epirubicin [1]. This molecule possesses a therapeutic effect similar to that of the parent compound but at doses producing equivalent antitumor effect, less cardiac toxicity results from epirubicin than from doxorubicin. In the present study it was necessary to treat mice with doses of epirubicin higher than those previously employed with adriamycin to obtain similar cardiac lesions [3]. However, the histopathologic appearance of the myocardial tissue was identical to that previously described for doxorubicin [3]. Sodium cromoglycate was extremely active in limiting the cardiotoxicity induced by adriamycin, and was equally efficacious in preventing the cardiotoxic action of epirubicin; this pharmacologic effect of sodium cromoglycate can probably be ascribed to its anti-exocytotic action. The antiallergic agent could also interfere with the pharmacokinetics of epirubicin and reduce cardiotoxicity by interfering with the

Table 1. Number of vacuoles in 0.042 mm^2 *of ventricular tissue, examined with a Zeiss MOP Videoplan image analyzer* (10 *fields per animal, five animals per treatment*)

Treatment	No. of vacuoles (mean ± 1 S.D.)
Epirubicin 8 mg/kg/week × 3 weeks	227.30 ± 22.44
Epirubicin 8 mg/kg/week × 3 weeks + cromolyn 200 mg/kg/week × 3 weeks	18.40 ± 3.92

amount of anthracycline taken up into the heart; however, the remarkable specificity of action of this compound and previous evidence indicating that histamine release is involved in the pathogenesis of acute and chronic anthracycline cardiotoxicity [4–7], suggests that inhibition of histamine release is the most likely mechanism.

These data further stress the importance of histamine release in the pathogenesis of anthracycline-induced cardiotoxicity, nevertheless it cannot be excluded that additional pathogenetic pre-existing causes proposed as basis for anthracycline cardiotoxicity, such as generation of free radical compounds [13], are involved.

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