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Doxorubicin is a potent inhibitor of interleukin 1 induced cartilage proteoglycan resorption in-vitro

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Abstract—Since interleukin 1 (IL-1) induces the transcriptional synthesis of enzymes responsible for cartilage resorption it was decided to examine the effects of the antitumour drug, doxorubicin, a DNA transcriptional inhibitor, on αIL-1-induced cartilage—resorption in-vitro. Doxorubicin inhibited the resorption in a concentration-dependent fashion, an effect which was shown to be reversible. Fine structure of the chondrocytes was preserved by the doxorubicin treatment with IL-1 in contrast to the extensive cellular destruction evident in cartilage treated with IL-1 alone. [^{14}C]doxorubicin was bound to cartilage proteoglycans, and this effect was promoted by treatment of the cartilage with IL-1. This binding of the drug may prevent access of the proteoglycans to destructive enzymes during the resorptive process induced by IL-1.

Interleukin 1 (IL-1) is among the important mediators of chronic inflammation present in synovial fluids of patients with rheumatoid arthritis (Bendtzen et al 1985) which induces destruction of cartilage and bone at articular surfaces (Saklatvala et al 1984; Sedgwick et al 1984; Krakauer et al 1985; Pettipher et al 1986; Dingle et al 1987). Of a wide range of pharmacological agents examined so far, only aminoquinoline and related antimalarials, and inhibitors of nucleic acid replication inhibit IL-1 induced cartilage proteoglycan resorption in-vitro; the conventional non-steroidal and steroidal anti-inflammatory drugs having little or no effects (Rainsford 1985a, b, 1986).

It was previously found that the DNA intercalator, echinomycin, inhibited IL-1-induced cartilage resorption, whereas its structural analogue TANDEM, which is only a weak intercalator, failed to inhibit the destructive effect of IL-1 (Rainsford 1985a). This, together with results of other pharmacological agents (Rainsford 1985a, b, 1986) suggested that a central event in mediating the cartilage destructive effects of IL-1 might involve DNA transcription. It was, therefore, decided to investigate the effects of the antitumour agent, doxorubicin, a drug which, though having multiple actions (Barranco 1984) is among the more successful of these agents (British National Formulary 1983; Weber-Stadelmann 1983). A further reason for studying the actions of this drug is its strong binding to sulphated mucopolysaccharides (Menozzi & Arcamone 1978), and as sulphated cartilage proteoglycans might be expected to likewise be expected to bind this water-soluble drug it might be expected that this property could protect the cartilage macromolecules from destruction by degradative enzymes whose production is stimulated by IL-1.

Methods

Cartilage culture. Bovine nasal septum cartilage from freshly (< 1 h) slaughtered adult cattle was dissected under sterile conditions and transverse sections (2·5 mm thick) cut from which discs (2 mm in diameter) were subsequently obtained using a sterile leather punch. The cartilage discs were pre-incubated for 1–2 days in Microtiter 96 well dishes in the presence of 200 μL Dulbecco's modified Eagle's medium (DMEM) containing 5% foetal calf serum (FCS), penicillin (200 μ mL^{-1}), and streptomycin (200 μ mL^{-1}) which were incubated in an atmosphere of 5% CO_2 in air (Saklatvala et al 1984). The medium was then changed and doxorubicin (0·5–100 μM) as the hydrochloride salt (Farmitalia Carlo Erba, Milan, Italy; Batch 4016DO50) was added together with chromatofocussed pI 4·8 porcine leucocyte α-IL-1 (gift of Dr. J. Saklatvala, Strangeways Research Laboratory, prepared as described by Saklatvala et al 1984), or human recombinant r-α-IL-1 (Genzyme Corp., Boston, MA, USA). Control cultures had medium along with or without IL-1 added. Sufficient IL-1 (ca 20 LAF units mL^{-1}) was added to give at least a four-fold increase in proteoglycan degradation, i.e. 80–90% above that in control cultures. Incubations with drug or solvent mixtures were performed in octuplicate for 4 days. Control cultures (without IL-1) had drugs alone.

Actinomycin D (0·5 and 0·04 μM, Sigma) was added in place of doxorubicin in some cultures to compare the effects of this conventional inhibitor of DNA synthesis on IL-1 induced cartilage resorption. Drug cytotoxicity was determined by comparing the release of glycosaminoglycans (GAGs) (see below) from those cultures to which the drugs or control (medium alone) mixtures had been added, with GAG release in controls (in DMEM ± 5% FCS).

Cartilage proteoglycan degradation was determined by measurement of the content of GAGs in the media compared with that in papain-digested cartilage (Saklatvala et al 1984). The assay of GAGs was performed using the dimethyl-methylene blue (Serva) technique of Farndale et al (1982). The drugs used did not interfere with this assay.

The effects of prior treatment with doxorubicin on pig α-IL-1 induced cartilage resorption were studied to determine if the effects of this potentially cytotoxic agent are reversible. In these studies freshly prepared cartilage discs were pre-incubated for 24 h in 1·0 and 0·5 μM doxorubicin, then briefly washed three times in sterile phosphate-buffered saline (PBS), before being incubated for 4 days in the presence of pig α-IL-1 (as above) in the absence of the drug. The release of GAGs from the cartilage discs was then calculated as above.

Uptake of [^{14}C-]doxorubicin. Bovine nasal cartilage discs (previously incubated for 2 days in control media as above) were incubated with 20 μM and 4 μM [^{14}C-]doxorubicin (batch No. LM 4835/38B, Farmitalia Carlo Erba, Milan, Italy) for 1 and 4 days in the presence and absence of human recombinant α-IL-1 (10 u mL^{-1}; National Institute for Biological Standards and Control batch 86/632). After washing in saline, the discs were individually frozen at −20°C. Half of these discs were sliced as thinly as possible with a prechilled scalpel while placed in a stainless steel dish on dry ice, and the proteoglycans in the pooled slices extracted by treatment for two days at 4°C with 4 M guanidine hydrochloride in 0·1 M sodium acetate buffer and 10 mM EDTA Na_2, pH 5·8, with a mixture of protease inhibitors comprising 100 mg L^{-1} trypsin inhibitor (Sigma), 5 mM benzamidine hydrochloride (Sigma) and 0·1 M ε-amino-caproic acid. The extracts free of residual sliced material were then dialysed twice for 2 days each against 0·1 sodium acetate with 10 mM EDTA Na_2, pH 5·8, freeze-dried and the dried fractions dissolved in 1 mL of the acetate buffer. The concentration of GAGs was determined in 100 μL aliquots of the mixture using the dimethyl methylene blue technique (Farndale et al 1982) and the radioactivity in another aliquot determined by scintillation counting after solubilization in Soluene (Packard).

The remaining groups of discs were individually digested for 2

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h at 65°C in 1 mL of 2 mL mL^{-1} papain (Sigma Cat. No. P3125) in 3·26 mg mL^{-1} of *N*-acetyl cysteine and 7·44 mg mL^{-1} EDTA Na_2 prepared in 0·05 M sodium phosphate buffer, pH 6·5. Aliquots (100 mL) of the digests were then added to triton-toluene scintillant for radio-active assay by scintillation counting. The concentration of GAGs was determined in another 100 μL of the digest.

Electronmicroscopy. To determine if treatment of the chondrocytes with doxorubicin affected their viability, ultrastructural observations were performed on cartilage discs which had been incubated with pig α-IL-1 for four days (using the same system described in culture methods above) in the presence and absence of the high concentration of 100 μM doxorubicin. The cartilage pieces were sliced into sections of approximately 0·5–1·0 mm and fixed for 4–6 h in 2% glutaraldehyde in 0·1 M sodium phosphate buffer pH 7·4 at room temperature (20°C). The tissues were then washed 4× in the buffer alone, post-fixed for 1 h in 1% w/v osmium tetroxide prepared in the phosphate buffer, and dehydrated in 30%, 90% and absolute ethanol, followed by 1,2-epoxypropane before embedding in araldite (Agar). Thick sections were stained with 1% w/v toludine blue in 1% w/v sodium borate for orientation by light microscopy from which those thin sections examined by electronmicroscopy (EM) were stained with lead citrate and uranyl acetate. The entire cartilage slice was examined for the presence of intact chondocytes and their morphology.

Results and discussion

Doxorubicin caused a concentration-related reduction in the release of GAGs from bovine nasal cartilage discs induced by porcine α-IL-1 (Table 1) and similarly by the human recombinant α-IL-1 (data not shown). The IC50 value for the inhibitory effect of doxorubicin being 0·8 μM in the presence of both the interleukins. Preincubation for 24 h with doxorubicin at concentrations around the IC50 value followed by PBS washing and treatment with the human recombinant α-IL-1 resulted in maximal resorption (Table 1). It therefore appears that the inhibitory effects of doxorubicin are reversible.

Inhibition of pig α-IL-1-induced cartilage resorption was also evident with actimomycin D (Table 1). Since doxorubicin acts like actinomycin D in inhibiting DNA dependent RNA synthesis (Barranco 1984) it is possible that the inhibitory effects of doxorubicin, though less potent than those of actinomycin D, could be mediated by the transcriptional effects of these drugs. The lower potency of doxorubicin compared with actinomycin D could, however, be due to binding of an appreciable proportion of the drug to proteoglycans, thus enabling only an appreciably lower proportion of the drug to interact with DNA. As shown in Table 2 a large proportion of the total ^{14}C-labelled drug added to the culture medium appears to be associated with, or bound to, extracted proteoglycans by four days culture. Interestingly, treatment of the cartilage with IL-1 appears to enhance the amount of doxorubicin which is bound to extractable proteoglycans at four days, though the total uptake into cartilage is somewhat lower with IL-1 than without this cytokine (Table 2). These differences might be related to complexities of the degradative effects of IL-1 on cartilage proteoglycans and the concomitant inhibition of this by the drug such that the denominator values for GAGs are proportionately altered. Whatever the basis the net effect is that IL-1 treatment promotes the binding of doxorubicin. This effect might be due to the cartilage-degrading enzymes produced in response to IL-1 treatment opening up sites on proteoglycan molecules for binding of the drug.

Table 1. Effects of doxorubicin on interleukin 1 induced resorption of bovine nasal cartilage in-vitro.

Treatment		% Release of GAGs (mean ± s.d.) with IL-1	% Release of GAGs (mean ± s.d.) No IL-1	Reduction %
Pig IL-1				
Doxorubicin	100 μM	9·3 ± 4·4*	10·2 ± 3·2	100
	50 μM	12·0 ± 4·3*	9·7 ± 4·6	96·7
	5 μM	15·8 ± 6·9*	10·4 ± 5·0	92·2
	0·5 μM	66·6 ± 24·4*	23·7 ± 16·8	38·4
Actinomycin D	0·5 μM	10·5 ± 2·0*	11·3 ± 6·8	100
	0·04 μM	14·3 ± 5·4*	13·0 ± 4·1	100
Control		94·9 ± 1·0	13·5 ± 5·2	
Human recombinant IL-1 Preincubation with Doxorubicin	1 μM	93·0 ± 1·6	16·8 ± 5·6	
	0·5 μM	86·0 ± 10·9	17·9 ± 6·9	
Control		92·0 ± 7·5	22·0 ± 7·5	

*Statistically significant reduction compared with IL-1 control (Student's *t*-test, $P < 0·05$). The extent of cartilage proteoglycan resorption was determined by measuring the concentration of glycosaminoglycans (GAGs) in the medium compared with that in the papain-digested cartilage as described in the methods. The percent release of GAGs thus determined represents the extent of cartilage resorption.

Since no significant increase in percent release of GAGs was evident in drug-treated control cultures without IL-1 added this is suggestive of their being no cytotoxic effects of the drugs as is evident with agents such as the retinoids.

Table 2. Uptake into cartilage and association with proteoglycans therein of ^{14}C-doxorubicin in-vitro.

Concn [^{14}C]doxorubicin added		Incubation Time	Concn in Cartilage Component with IL-1 nmol mg^{-1} GAGs	Total	No IL-1 nmol^{-1} GAGs	Total
μM	nmol total	(days)	(mean ± s.d.)	drug (%)	(mean ± s.d.)	drug (%)
Proteoglycan-associated drug						
20	4·0	1	0·34 ± 0·14(b)	8·5	0·31 ± 0·05(d)	7·8
4	0·8		0·50 ± 0·24(b)	62·5	0·29 ± 0·01(d)	36·3
20	4·0	4	2·47 ± 2·01(a)	61·8	0·74 ± 0·51(c)	18·5
4	0·8		1·43 ± 0·99(a)	179	0·42 ± 0·01(c)	52·5
Total in cartilage (papain digested)						
20	4·0	4	0·24 ± 0·09(b)	6·0	1·22 ± 1·01(b)	30·5
4	0·8		0·29 ± 0·26(b)	36·3	0·42 ± 0·31(b)	52·5

N = 8 (a), 4 (b), 3 (c) or 2 (d) for group.

Percent total drug refers to the concentration of drug in the extractable proteoglycans (PrGns) or disc in nmol mg^{-1} GAGs of disc or PrGns compared with the total added to the culture medium (in nmol).

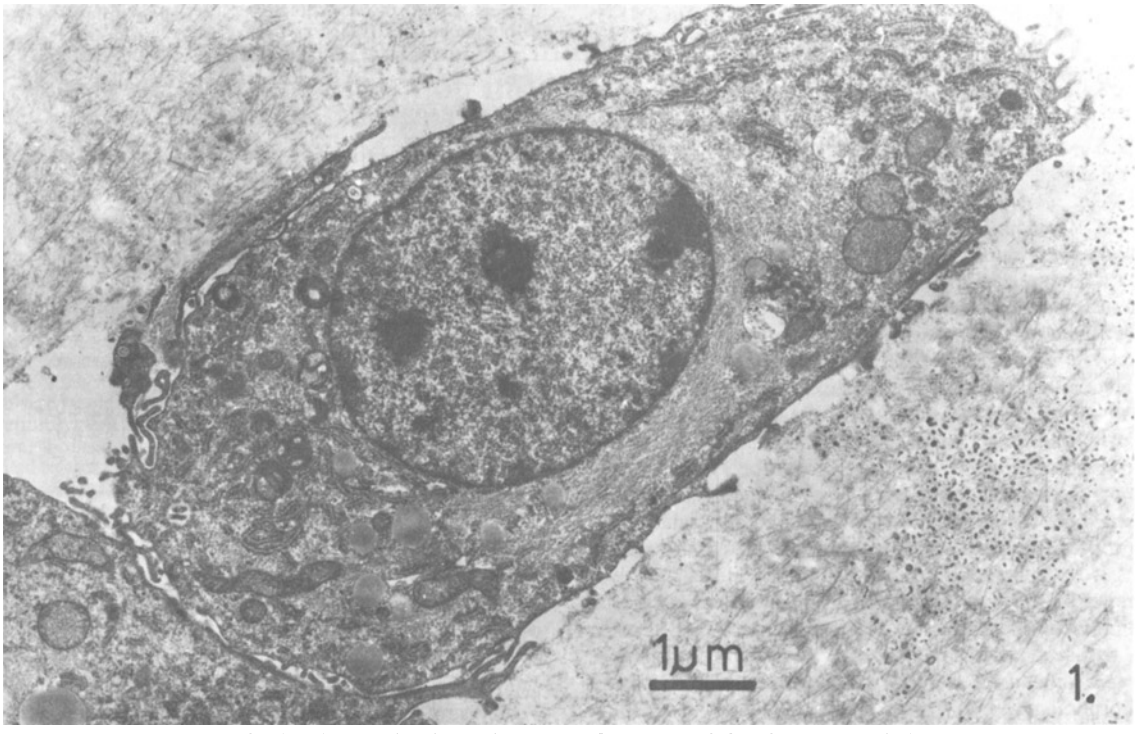

FIG. 1. Fine structure of a chondrocyte showing maintenance of near normal chondrocyte morphology upon treatment with 100 μM doxorubicin in combination with IL-1.

Electron microscopic observations of the chondrocytes in cartilage cultured in the presence of pig α-IL-1 for four days revealed an extensive number of cells which were filled with large numbers of dense lipid-like vesicles and exhibited extensive disruption or even disintegration (Fig. 1). Such morphology suggested extensive celular destruction which might have been produced by the destructive enzymes produced in response to the treatment with IL-1. Treatment of cartilage with the highest concentration of doxorubicin in combination with the IL-1 resulted in maintenance of the near normal morphology of the chondrocytes (Fig. 2). This striking preservation of chondocyte morphology suggests that by blocking the transcriptional effects of IL-1 doxorubicin prevents the production of not only those enzymes responsible for degradation of proteoglycans but also those in cellular destruction. The physical association of doxorubicin with proteoglycans may also have consequences for preventing enzymic attack for proteoglycans. Clearly, the reversible effects of doxorubicin on IL-L-induced cartilage resorption suggests that a mode of action of the drug on DNA dependent replicative events might be reversible.

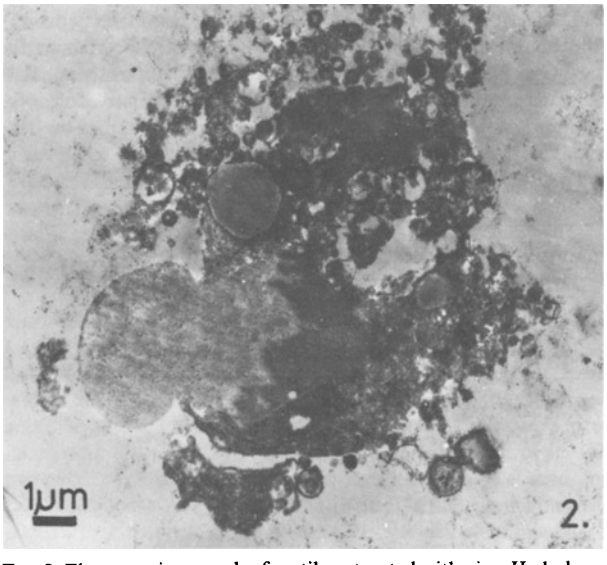

FIG. 2. Electronmicrograph of cartilage treated with pig α IL-1 alone showing the extensive cellular destruction and accumulation of lipid-like vacuoles in the remnant of a chondrocyte. Nearly all the cells in the cartilage so treated with IL-1 for four days show variants of this morphology indicative of extensive cellular disruption and cytolysis.

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Difference spectrophotometric assay of nitrazepam in tablet formulations

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Abstract—A difference spectrophotometric procedure is described for the assay of nitrazepam in tablet formulations. The method is based on the measurement of absorbance at 282 nm of a solution of the tablet extract in 0·1 M hydrochloric acid (pH 1) relative to that of an equimolar solution in 0·1 M sodium hydroxide (pH 13). The method is precise and selective for nitrazepam in the presence of the tablet excipients and 2-amino-5-nitrobenzophenone, the principal hydrolysis product of nitrazepam. The absence of a constant isosbestic point in the difference spectrum of nitrazepam during hydrolysis in alkaline solution indicates the presence of a previously unrecognized intermediate hydrolysis product.

The advantages of difference spectrophotometry, which is a modified spectrophotometric technique for the selective analysis of certain drugs in the presence of absorbing substances that interfere in a direct spectrophotometric assay, have been amply demonstrated (eg Doyle & Fazzari 1974; Davidson 1976, 1982, 1984a, 1987). The basis of a difference spectrophotometric assay is that an absorbance difference (ΔA) is measured between two equimolar solutions of the absorbing analyte in the presence of different reagents that reproducibly alter the spectral properties of the analyte. Provided that the absorbance of the other absorbing interferents is not affected by the reagents, their ΔA is zero and their contribution to the measured ΔA of the sample is eliminated. Of the many reagents that have been used to induce differences in the spectra of drugs, simple aqueous acids, alkalis and buffers have been the most frequently used because many drugs are weak acids or bases whose state of ionization and absorptivity depend on the pH of the solution.

The 1,4-benzodiazepines comprise a class of drug in which several sites of protonation and deprotonation exist, and which exhibit marked changes of spectral properties as a result of variation of pH (Barrett et al 1973). These spectral transformations have been utilized in the simultaneous difference spectrophotometric assay of chlordiazepoxide and its hydrolysis product, demoxepam (Davidson 1984b) and in the assay of diazepam, clonazepam and medazepam (Abdel-Hamid et al 1984). As part of a continuing research programme, the present study was undertaken to develop a difference spectrophotometric assay for nitrazepam and to evaluate its suitability as a stability-indicating assay for the measurement of the reaction kinetics of the hydrolysis of nitrazepam in alkaline solution.

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The principal decomposition reaction of the 1,4-benzodiazepines is hydrolysis to the corresponding benzophenone by cleavage of the 1,2-amide and 4,5-azomethine bonds (Schutz 1982). Several of the benzodiazepines yield an intermediate hydrolysis product: diazepam produces 5-chloro-2-glycyl (methyl)amino-benzophenone in acidic solution by the preferential cleavage of the 4,5-bond whereas oxazepam yields an acidic intermediate product by the preferred cleavage of the 1,2-bond (Han et al 1977a). It has been reported also that the initial hydrolysis step of nitrazepam is at the 4,5-bond and not the 1,2-amide linkage, attributed to a preferential activation for hydrolysis of the azomethine linkage by the nitro group. The intermediate hydrolysis product was detected only in solutions of pH less than the pK_a of nitrazepam, 3·2 (Han et al 1977b).

On the basis of the facts known about the hydrolysis of nitrazepam, it was considered during the initial development of the method that a stability-indicating assay for the measurement of the reaction kinetics of nitrazepam in alkaline solution should be selective for nitrazepam in the presence of 2-amino-5-nitrobenzophenone only. The results of the study showed that although the method was selective for nitrazepam in the presence of the benzophenone, an extra product was present in solutions of nitrazepam hydrolysed at alkaline pH that interfered in the assay.

Materials and methods

Materials. Nitrazepam and 2-amino-5-nitrobenzophenone were gifts from Roche Products Ltd. Hydrochloric acid, 1 M, sodium hydroxide, 1 M, acetate buffer pH 6, 0·1 M and sodium tetraborate, 0·01 M, were prepared from analytical reagent grade substances.

Spectrophotometer. Absorption and difference absorption spectra were recorded in 1 cm silica quartz cells over the range 230–450 nm by using a Perkin Elmer 552 ultraviolet-visible double-beam recording spectrophotometer. The spectral bandwidth was 2 nm, the scan speed 1 nm s^{-1} and response 0·5 s.

Standard solutions. A standard solution of nitrazepam (200 μg mL^{-1}) was prepared by dissolving 20 mg in 10 mL of ethanol and diluting it to 100 mL with water. A 5 mL aliquot was transferred to two 100 mL volumetric flasks containing 10 mL of 1 M