Finally, we examined the effect of CPZ on collagen synthesis in clone MC3T3-El cells (Table 2). CPZ caused a decrease in collagen synthesis dose-dependently with concentrations up to 20 μg/ml. Non-collagen protein synthesis was also lowered, but its effect on synthesis of collagen was greater than that on non-collagen. Taken together, these findings indicate that CPZ suppresses osteoblastic cell function *in vitro*. These *in vitro* and *in vivo* findings suggest that CPZ lowers osteoblastic cell function, reflecting on suppression of bone formation. However, we do not know now the mechanism of CPZ activity on osteoblastic cells.

Although CPZ is not used for women of child-bearing age because of its suspected teratogenetic side effects [4], this drug (1–4 mg/kg) is still used for treatment of emotionally disturbed subjects. These therapeutic doses are sufficient to suppress both alkaline phosphatase activity and collagen synthesis in osteoblastic cells *in vitro*. And the inhibitory effects of CPZ on bone formation *in vivo* as well indicate caution in its application in man. In fact, a single injection of CPZ (2.5 mg/kg) affected alkaline phosphatase activity in rat calvaria, and the effect of CPZ was more specific and long-term for bone.

In summary, CPZ specifically lowered alkaline phosphatase activity in rat calvaria *in vivo*. This agent also suppressed alkaline phosphatase activity and collagen synthesis in osteoblastic cells *in vitro* in a dose-related fashion, both of these levels were one-half those of controls at a concentration of 10 μg/ml. These *in vivo* and *in vitro* findings suggest an inhibitory effect of CPZ on bone formation via a suppression of osteoblastic cell function.

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Inhibition of prostaglandin biosynthesis by the mast-cell-degranulating agent compound 48/80 but not by the mast-cell-degranulating peptide (peptide-401) from bee venom

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Peptide-401 [1] (MCD-peptide [2]), the mast-cell-degranulating peptide from bee venom, is potently anti-inflammatory in several experimental models of inflammation in the rat [3]. The peptide is 100 times more active than hydrocortisone in the carrageenin hind paw oedema assay and attenuates or completely suppresses the inflammation associated with lesions in rat adjuvant arthritis, depending on whether the peptide is administered after or before establishment of the disease. We have also shown that a second potent degranulator of rat peritoneal mast cells, the synthetic compound 48/80, shows anti-inflammatory activity in rat carrageenin oedema comparable to that of peptide-401 [4].

It has been reported that peptide-401 was able to inhibit the synthesis of PGE_2, PGD_2 and $PGF_{2\alpha}$ by prostaglandin synthetase isolated from sheep seminal vesicles [5]. Although the concentrations of peptide required to show significant inhibition ($ED_{50} \approx 50$ mM) were higher than those likely to be achieved *in vivo* with the low doses required for anti-inflammatory activity, it was found (using fluorescently-labeled peptide-401) that binding of the peptide in a mixed cell population was relatively specific for polymorphonuclear leukocytes (PMN). These cells mediate the inflammatory response to carrageenin in the rat over those phases (1–5 hr) responsive to the anti-inflammatory action of peptide-401 and compound 48/80 [6] and are the major source of prostaglandins in this model of inflammation [6, 7]. The possibility arises that interference in prostaglandin biosynthesis or some other response of PMN elicited to the inflammatory site might contribute to the anti-inflammatory activity of peptide-401 and compound 48/80. We have therefore isolated casein-elicited rat peritoneal PMN and tested the effects of compound 48/80 and peptide-401 on the release of prostaglandins and other potential inflammatory mediators (lysosomal enzymes and superoxide anions) from these cells during phagocytosis.

Materials and methods

Peptide-401 was purified from bee venom as described by Banks *et al.* [8]. Other venom components having potential

pharmacological activity (melittin, phospholipase A2 and hyaluronidase) were shown to be present at less than 0.2% by weight by cross immunoprecipitation with the respective antisera. These assays were performed by Dr. D. M. Kemeny, Guys Hospital Medical School, London. Compound 48/80 was from Sigma Chemical Co. (Poole, Dorset) and its concentrations were calculated using $MW_{av} = 1000$.

Male Sprague–Dawley rats (300–600 g) were injected (i.p.) with 10 ml sterile sodium casein (4% w/v, pH 7.4) and were killed after 18 hr by CO_2 intoxication. Peritoneal cells were recovered in phosphate-buffered saline (PBS) containing 40 U ml^{-1} preservative-free heparin, washed and resuspended in Geys balanced salt solution (GBSS; Gibco Ltd., Paisley, Scotland) at 3×10^7 cells ml^{-1}. A differential cell count showed that 80% of the cells were PMN with the remainder being mostly lymphocytes; the numbers of macrophages or erythrocytes were negligible.

To stimulate the phagocytic response, PMN were incubated with killed yeast particles opsonised with complement from fresh human serum. A ratio of yeast particles to cells of 50 : 1 gave optimal release of prostaglandins. Opsonised yeast (400 μl; 1.5×10^9 particles ml^{-1}) and drug (2 μl in dimethylformamide) were made up to 1.6 ml with GBSS at 37°. An aliquot of cell suspension (400 μl; 1.2×10^7 cells per assay) was added and the mixture incubated at 37° for 30 min with gentle shaking (in some experiments the cells were preincubated for 1 hr with the drug to be tested). The incubation was stopped by centrifugation and the supernatant was removed, acidified to pH 3 with citrate and the prostaglandins extracted into ethyl acetate. The residue obtained after evaporation under nitrogen was stored at −20° until assay for prostaglandins.

Prostaglandins were determined by radioimmunoassay using antisera from the following sources: PGE_2 from Sigma and Mannheim Boehringer (Lewes, Sussex); TXB_2 (the stable metabolite of thromboxane) from Roche Products Ltd. (Welwyn Garden City, Herts.); 6-oxo $PGF_{1\alpha}$ (the stable metabolite of prostacyclin) from Instit Pasteur (Marnes la Coquette, France) and Roche. Prostaglandins were from Roche, Sigma and Upjohn Chemical Co. (Kalamazoo, MI, U.S.A.). Radiolabeled prostaglandins were from Amersham International (Amersham, Bucks.) or Upjohn Chemical Co. The assays were performed in triplicate according to the protocol supplied by the manufacturer of each antiserum. Indomethacin, an inhibitor of prostaglandin cyclooxygenase activity, was used as a control. Generation of all the prostaglandins measured by radioimmunoassay was inhibited by indomethacin at 10^{-4} M (PGE_2 by 99%, 6-oxo $PGF_{1\alpha}$ by 90% and TXB_2 by 95%). PGE_2 generated by PMN was also determined by bioassay on the rat stomach fundus as described by Vane [9]. The response of the muscle was calibrated repeatedly using standard PGE_2.

To assay superoxide anions generated from phagocytosing PMN, incubations were carried out as described above with the following additions. Before addition of the cells the yeast particles were preincubated with 50 μl catalase (2000 Sigma units ml^{-1} in GBSS) and 500 μl ferricytochrome-c (225 μM). After incubation and collection of the supernatant, the conversion of ferri- to ferrocytochrome-c was determined by u.v. spectroscopy using $\Delta\varepsilon_{370} = 21.0 \times 10^3 M^{-1}cm^{-1}$ [10]. Superoxide-independent reduction of ferricytochrome-c (determined from assays containing superoxide dismutase and typically less than 15% of the total reducing activity) were subtracted to give the values in Fig. 2. β-Glucuronidase activity in incubation supernatants was determined from the enzymatic release of phenolphthalein from phenolphthalein-β-D-glucuronic acid [11].

Results and discussion

Compound 48/80 inhibited the synthesis of prostaglandins by phagocytosing rat PMN (Fig. 1). Each of the cyclooxygenase prostaglandins was inhibited by compound 48/80 at 2.5×10^{-5} M (PGE_2 by 25%, 6-oxo-$PGF_{1\alpha}$ by 30% and TXB_2 by 50%) while at 2.5×10^{-7} M, TXB_2 generation was still inhibited by 35%. The anti-prostaglandin activity occured without affecting the other measured responses of phagocytosing PMN (Fig. 2), indicating that compound 48/80 is a selective inhibitor of prostaglandin synthesis in rat PMN. It has recently been reported [12, 13] that compound 48/80 is a potent and selective antagonist of calmodulin-dependent functions and this may be relevant to the effect of the drug on prostaglandin biosynthesis reported here. The rate-limiting step in prostaglandin biosynthesis is the generation of fatty acid (arachidonate) from membrane phospholipids by phospholipase A_2 which is a calmodulin dependent process [14]. Although the action of compound 48/80 and (peptide-401) in stimulating the degranulation of rat peritoneal mast cells requires the *activation* of calmodulin-dependent enzymes involved in the secretory process (phospholipase A_2, adenylate cyclase and phosphodiesterase) [15], the respective actions of compound 48/80 are not contradictory. In the case of the mast cell, compound 48/80 acts by stimulation at the cell surface [16] without having access to the cell interior where antag-

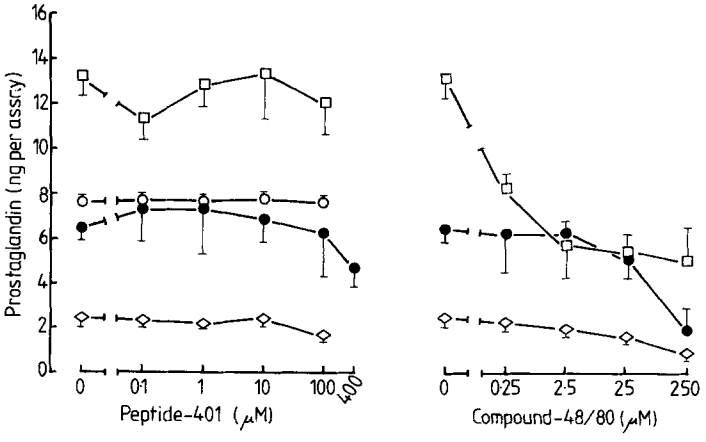

Fig. 1. Effect of peptide-401 and compound-48/80 on the generation of PGE_2 ○, 6-oxo-$PGF_{1\alpha}$, PGF_1 ◇ and TxB_2 □ from phagocytosing PMN. Open symbols are determinations by radioimmunoassay; closed circles denote bioassay. Each point is the mean of three determinations. In all cases 1.2×10^7 cells were used per assay.

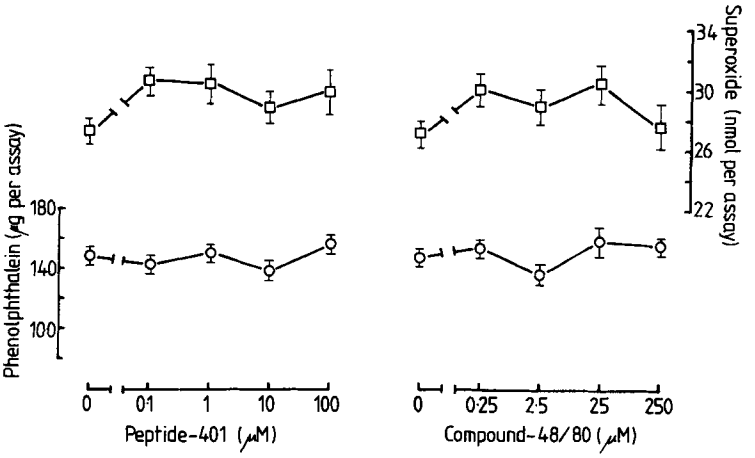

Fig. 2. Effect of peptide-401 and compound-48/80 on the release of lysosomal enzyme (represented by β-glucuronidase activity) ○ and superoxide □ from phagocytosing PMN. Each point is the mean of three determinations. In all cases 1.2×10^7 cells were used per assay.

onism of calmodulin activity could occur. In the PMN, compound 48/80 probably acts inside the cell (gaining entry during the phagocytic process) to act on *de novo* prostaglandin synthesis by calmodulin antagonism or some other mechanism. Thus the antagonism of prostaglandin synthesis was not enhanced by preincubating the cells with the drug, and cell-surface responses (such as superoxide production) were not affected.

The question arises of the significance of the inhibitory effects of compound 48/80 on prostaglandin biosynthesis *in vivo*. Doses of the compound required to attenuate carrageenin oedema ($ED_{50} \approx 10$ mg kg^{-1}) are about 10-fold greater than those at which peptide-401 is effective [17] and are sufficiently large that inhibition of prostaglandin synthesis might be expected to contribute to the pharmacological effects of compound 48/80. However the attenuation of carrageenin oedema by compound 48/80 and peptide-401 follow very similar courses and are affected in the same way by a variety of pharmacological interventions (such as mast cell depletion or treatment with histamine and serotonin antagonists [4, 17]), indicating that the two compounds act by similar mechanisms. The lack of effect of peptide-401 on the production of cycloxygenase prostaglandins (Fig. 1) indicates that these mechanisms do not include the inhibition of prostaglandin synthesis. Indeed, this study underlines the ineffectiveness of the suppression of cyclooxygenase prostaglandins as a basis for attentuation of carrageenin oedema. Indomethacin, which completely suppresses prostaglandin synthesis at 10^{-4} M, is about 50-fold less potent against carrageenin oedema than peptide-401 [3, 18], which in turn is inactive as an inhibitor of prostaglandin synthesis. It seems unlikely, therefore, that the inhibition of prostaglandin biosynthesis makes a significant contribution to the anti-inflammatory activity of compound 48/80 although this effect should be considered when using the drug in pharmacological experiments where anti-prostaglandin activity may be important.

In summary, we have investigated the possibility that the inhibition of some response of phagocytosing PMN elicited to the inflammatory site might contribute to the anti-inflammatory activity of peptide-401 and compound 48/80. Compound 48/80 was found to inhibit the synthesis of cyclooxygenase prostaglandins from phagocytosing PMN *in vitro* at pharmacologically significant concentrations. However, the lack of similar activity by peptide-401 which appears to attenuate carrageenin oedema by the same mechanism(s) as compound 48/80, suggests that the anti-prostaglandin activity of compound 48/80 may not be important for its anti-inflammatory activity.

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Effects of ascorbic acid on biologically obtained diaziquone free radicals

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The antitumor activity of quinone-containing agents is believed to be influenced by their ability to undergo biological activation via a free radical mechanism involving their quinone moieties [1–4]. In the case of diaziquone (AZQ), it is possible that bioreductive alkylation is part of the mechanism of action of this drug [5, 6]. AZQ has been shown to be easily reduced to its free radical species by rat liver microsomes [4], by NADPH cytochrome *c* reductase [4] and by cells in culture [7]. The reduced AZQ thus obtained produces a five line electron spin resonance (ESR) spectrum which we have characterized by electrochemical reduction and ESR [8]. We report here the influence of ascorbic acid on the AZQ free radical generated by human erythrocytes and by L1210 murine leukemic cells.

Methods

Diaziquone (AZQ), 2,5-diaziridinyl-3,6-bis(carboethyoxyamino)-1,4-benzoquinone (see Fig. 1), was supplied by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD, U.S.A., and shown to be 99% pure by high pressure liquid chromatography [9]. L1210 murine leukemia cells were maintained *in vitro* by serial culture in RPMI 1640 medium, as previously described [7]. L1210 cells were harvested by centrifugation, washed twice with 0.15 M NaCl, and resuspended at a concentration of 10^7 cells/ml in calcium, magnesium free Hanks' balanced salt solution (HBSS), pH 7.5. Blood was obtained from human volunteers and anticoagulated with heparin (50 units/ml). Erythrocytes were separated by centrifugation at 900 *g*. The plasma and "buffy coat" were removed, and the cells were resuspended in 1 ml HBSS, treated with 1 mM AZQ, and subjected to electron spin resonance analysis. In some cases the "buffy coat" was not removed. Oxygen uptake was determined with a Clark-type electrode in a Biological Oxygen Monitor (model 53, Yellow Springs Instrument Co., Yellow Springs, OH). Oxygen consumption rates were calculated as previously described [4, 7]. The 1-ml reaction mixtures contained AZQ (1 mM), sodium borohydride ($NaBH_4$) as a reducing agent or ascorbic acid (AH_2). ESR spectra were obtained with an X-band (9.3 GHz) Varian E-109 Century Series spectrometer equipped with 100 kHz field modulation. A dual rectangular cavity was used which contained strong pitch (g = 2.0028) in one section and the sample in an ESR flat cell in the other. The concentration of the AZQ free radical was calculated by double integration with a Nicolet 1180 computer (Madison, WI) using 2,2-diphenyl-1-picrylhydrazyl as a standard [7]. AZQ was reduced to $AZQH_2$ with $NaBH_4$ at a 3 to 1 ratio of $NaBH_4$ to AZQ. AZQ solutions (1 mM) in phosphate-buffered saline (PBS), pH 7.5, became colorless after the addition of sodium borohydride (3 mM). Fifty microliters of this colorless solution was injected into a 1-ml PBS solution, pH 7.5, and the oxygen consumption from this solution was measured in the oxygen monitor described above. The final concentrations for the oxygen consumption measurements were 50 μM for AZQ and 150 μM for sodium borohydride respectively. Oxygen consumption evaluation was based on a 100% oxygen concentration of 200 μM, calculated for our conditions as in Ref. 4.

Results and discussion

Diaziquone free radicals were observed when the drug (1 mM final concentration) was added to a suspension of red blood cells (Fig. 1). These free radicals gave the same five line ESR spectra previously reported for the reduction of AZQ by microsomes, purified NADPH cytochrome *c* reductase [4] and L1210 murine leukemic cells [7]. If the "buffy coat" was only partially removed or not removed, a rapid decay of the AZQ free radical was observed with the appearance of a 1.8 G doublet (Fig. 1). This doublet was strongly suggestive of the ascorbyl radical, the presence of which was confirmed by the appearance of the same doublet when ascorbic acid, either as a solid or in solution (*ca* 2 mM), was added to a mixture of AZQ (1 mM) and L1210 cells which had reduced AZQ to its free radical anion (Fig. 2B). In preparations where the "buffy coat" was mixed in with the red blood cells, the ascorbyl radical appeared at various degrees of intensity and at various times after the addition of AZQ depending on the blood donor. The 16-min time in Fig. 1 is one of the fastest times observed. The "buffy coat" of a red blood cell preparation is rich in leukocytes which are second only to the adrenals in containing the most ascorbic acid in humans (250–350 mg/kg net wt) [10].

The most likely equations involving endogenous or exogenous monodehydro ascorbate (AH_2, ascorbic acid)* that can explain the observations above are equations 1–3. Ascorbic acid is oxidized to the ascorbyl radical ($\dot{A}H$) while the AZQ free radical ($AZ\dot{Q}H$) is fully reduced to the hydroquinone ($AZQH_2$) (Eq. 1). This is an electron transfer reaction which has been observed by Schuler for *p*-semiquinones [11] and by Borg *et al.* for a variety of free radicals including 6-hydroxy dopamine and dialuric acid [12]. In the latter case, the ascorbyl radical was always observed [12].

* We have chosen to write all chemical reactions in the protonated form for simplicity.