



# Chondroitin sulphate inhibits connective tissue mast cells

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**1** Mast cells derive from the bone marrow and are responsible for the development of allergic and possibly inflammatory reactions. Mast cells are stimulated by immunoglobulin E (IgE) and specific antigen, but also by a number of neuropeptides such as neurotensin (NT), somatostatin or substance P (SP), to secrete numerous pro-inflammatory molecules that include histamine, cytokines and proteolytic enzymes.

**2** Chondroitin sulphate, a major constituent of connective tissues and of mast cell secretory granules, had a dose-dependent inhibitory effect on rat peritoneal mast cell release of histamine induced by the mast cell secretagogue compound 48/80 (48/80). This inhibition was stronger than that of the clinically available mast cell 'stabilizer' disodium cromoglycate (cromolyn). Inhibition by chondroitin sulphate increased with the length of preincubation and persisted after the drug was washed off, while the effect of cromolyn was limited by rapid tachyphylaxis.

**3** Immunologic stimulation of histamine secretion from rat connective tissue mast cells (CTMC) was also inhibited, but this effect was weaker in umbilical cord-derived human mast cells and was absent in rat basophilic leukemia (RBL) cells which are considered homologous to mucosal mast cells (MMC). Oligo- and monosaccharides were not as effective as the polysaccharides.

**4** Inhibition, documented by light and electron microscopy, involved a decrease of intracellular calcium ion levels shown by confocal microscopy and image analysis. Autoradiography at the ultrastructural level showed that chondroitin sulphate was mostly associated with plasma and perigranular membranes.

**5** Chondroitin sulphate appears to be a potent mast cell inhibitor of allergic and nonimmune stimulation with potential clinical implications.

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**Keywords:** Chondroitin sulphate; disodium cromoglycate; heparin; histamine; mast cells; proteoglycans; secretion

**Abbreviations:** ChS, chondroitin sulphate; 48/80, compound 48/80; IgE, immunoglobulin E; NT, neurotensin; RBL, rat basophilic leukaemia; SP, substance P

## Introduction

Mast cells derive from the bone marrow and migrate into connective and mucosal tissues (Galli, 1993), where they are located at strategic points around capillaries close to nerve endings (Theoharides, 1996). Mast cells are critical for allergic reactions where the stimulus is immunoglobulin E (IgE) and specific antigen; however, there are also other nonimmune mast cell triggers that include anaphylatoxins, kinins, cytokines, as well as various neuropeptides (Baxter & Adamik, 1978; Coffey, 1973) such as somatostatin (Theoharides & Douglas, 1978), neurotensin (NT) (Carraway *et al.*, 1982) and substance P (SP) (Fewtrell *et al.*, 1982). When stimulated, mast cells synthesize and secrete numerous vasoactive, nociceptive and inflammatory mediators (Galli, 1993) that include histamine, kinins, prostaglandins, leukotrienes, cytokines, as well as the proteolytic enzymes chymase and tryptase (Schwartz, 1987). Such evidence has implicated mast cells also in inflammatory conditions (Galli, 1993; Theoharides, 1996). Sulphated proteoglycans constitute the major constituents of mast cell secretory granules. Of these, heparin is mostly found in connective tissue mast cells (CTMC), while chondroitin sulphate in MMC (Seldin *et al.*, 1985b; Razin *et al.*, 1983). Recent evidence indicates that chondroitin sulphate is also

present in human connective tissue (Krilis *et al.*, 1992) and umbilical cord-derived human mast cells (Blom *et al.*, 1996).

A number of compounds have been reported to block rat mast cell secretion experimentally. These include the lodoxomides (Johnson, 1980), doxantrazole (Pearce *et al.*, 1982; Fox *et al.*, 1988), quercetin (Fox *et al.*, 1988; Fewtrell & Gomperts, 1977) and certain histamine-1 receptor antagonists such as hydroxyzine (Theoharides *et al.*, 1985; Fischer *et al.*, 1995) and ketotifen (Nemeth *et al.*, 1987). The only clinically available 'anti-allergic' drugs are cromolyn (Orr *et al.*, 1971) and its structurally related nedocromil (Enerbäck & Bergström, 1989); yet, they are not capable of effectively blocking human mast cell secretion (Okayama *et al.*, 1992). In addition, the inhibitory action of these drugs is short-lived due to the induction of tachyphylaxis (Theoharides *et al.*, 1980) and they do not inhibit mucosal mast cells (MMC) (Barrett & Metcalfe, 1985; Pearce *et al.*, 1982; Fox *et al.*, 1988). Among mast cell derived molecules, histamine has an autoinhibitory action only in the brain through activation of histamine-3 receptors (Rozniecki *et al.*, 1999), while arachidonic acid products have variable and weak inhibitory effects (Theoharides, 1990). We hypothesized that chondroitin sulphate released from inflamed tissue, as well as from mast cells, could have an inhibitory effect on mast cell secretion. Here we show that chondroitin sulphate is a potent inhibitor of histamine release from CTMC.

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## Methods

### *Mast cell purification and treatment*

Peritoneal mast cells were isolated from male (250–300 g) Sprague Dawley rats (Jackson Labs, Bar Harbor, ME, U.S.A.) using Locke's solution (mM: NaCl 150, KCl 5, HEPES 5, CaCl<sub>2</sub> 2, 0.1% bovine serum albumin, 0.1% Dextrose, pH 7.2) and were purified (>95%) by centrifugation through 22.5% Metrizamide (Accurate Chemical & Scientific Corp., Westbury, NY, U.S.A.) (Theoharides *et al.*, 1982); the remaining cells were lymphocytes and a few macrophages. The cell pellet was washed and resuspended (10<sup>5</sup> cells ml<sup>-1</sup>) in Locke's solution. Following the indicated incubation times with either disodium cromoglycate, chondroitin sulphate C from shark cartilage or the mono- and disaccharides (Sigma, St. Louis, MO, U.S.A.), the cells were washed by centrifugation at 350 × *g* for 5 min at room temperature and resuspended in Locke's solution. The chondroitin sulphate was reported to be over 90% pure type C, balanced with type A. In some cases, chondroitin sulphate and <sup>3</sup>H-chondroitin sulphate (see below), was dissolved in 40 mM NaCl to dissociate any loose interaction with other smaller molecules and was then filtered and washed through a 50K exclusion filter (Millipore, Bedford, MA, U.S.A.) to remove any such molecules. Mast cells were stimulated with either: (a) 0.1 μg ml<sup>-1</sup> compound 48/80 (48/80); (b) 5 × 10<sup>-5</sup> M SP in Locke's solution without Ca<sup>++</sup> plus 0.1 mM EDTA (to chelate any extracellular calcium) for 30 min; (c) immunologically (see below).

### *Culture of RBL cells*

The rat basophilic leukaemia (RBL) cells are considered homologous to rat mucosal mast cells (Seldin *et al.*, 1985a). They were kindly provided by Dr Henry Metzger (NIH) and were grown as described previously (Isersky *et al.*, 1975).

### *Isolation and culture of umbilical cord-derived human mast cells*

Human umbilical cord blood samples were obtained from normal full-term deliveries under the approval from the Human Investigation Review Board. Cord blood was collected in tubes containing 10 U ml<sup>-1</sup> heparin and was diluted with twice the volume of phosphate-buffered saline (PBS). Mast cell progenitors were separated as described before (Kempuraj *et al.*, 1999). Mononuclear cells were separated by density-gradient centrifugation using Lymphocyte Separation Medium (LSM; Organon Teknika Corp, Durham, NC, U.S.A.). The interface containing mononuclear cells was collected and CD34<sup>+</sup> cells were separated using a magnetic separation column according to the manufacturer's instructions (Macs II, 441-01; Miltenyl Biotec, Bergisch Gladbach, Germany). Magnetic microbeads were removed with the CD34<sup>+</sup> isolation kit (Miltenyl Biotec). The CD34<sup>+</sup> cells were further separated with mouse anti-CD34 antibody followed by antimouse IgG1 magnetic microbeads (Miltenyl Biotec). The CD34<sup>+</sup> cells were cultured in Iscove's Modified Dulbecco's Medium (Gibco BRL, Grand Island, NY, U.S.A.) supplemented with 10% FBS, 100 ng<sup>-1</sup>ml human stem cell factor (SCF) from Amgen (Thousand Oaks, CA, U.S.A.) and 50 ng ml<sup>-1</sup> IL-6 (Atlanta Biologicals, Norcross, GA, U.S.A.) in 75-cm<sup>2</sup> flasks at 37°C in 5% CO<sub>2</sub>, for greater than 10 weeks (Kinoshita *et al.*, 1999).

### *Immunologic stimulation of mast cells*

Unpurified rat peritoneal mast cells (10<sup>6</sup> ml<sup>-1</sup>) were first incubated with 1.5 μg/10<sup>3</sup>cells ml<sup>-1</sup> rat IgE (Zymed, So. San Francisco, CA, U.S.A.) for 30 min in order to occupy the respective receptors to protect them from destruction during the purification step (Coutts *et al.*, 1980); mast cells were then incubated with rabbit anti-IgE (3–15 μg/10<sup>4</sup> cells ml<sup>-1</sup>) (Zymed) for 30 min at 37°C. The reaction was stopped by centrifuging the cells at 350 × *g* for 5 min at 4°C.

Human mast cells were sensitized with human IgE (Chemicon International, Temecula, CA, U.S.A.) at 1.5 μg ml<sup>-1</sup> for 48–72 h. Then the cells were washed with Dulbecco's Phosphate Buffered Saline (Gibco, Grand Island, NY, U.S.A.) solution and Human Tyrode solution once in each. These cells were then challenged with anti-human IgE (Dako, Carpinteria, CA, U.S.A.) at 15 μg ml<sup>-1</sup> for 30 min with or without pretreatment with the test compound in a shaking water bath at 37°C. The supernatant was then collected by centrifugation at 350 × *g* for 5 min for the histamine release assay.

RBL-2H3 cells were stimulated as 1.5 × 10<sup>-5</sup> cells per well in 12-well plates. RBL cells were used at mid-logarithmic stage, day 2 of culture postplating, as shown on the growth curve (Trnovsky *et al.*, 1993). After 48 h, cells were washed twice with PBS and sensitized for 24 h with 1.0 μg ml<sup>-1</sup> of rat monoclonal IgE to dinitrophenol (DNP) (Zymed). Cells were washed twice with PBS and stimulated with 50 ng ml<sup>-1</sup> DNP-BSA (Sigma) for 30 min in 37°C.

For histamine secretion the supernatant and the cell pellet, to which an equal volume of Locke's or Tyrode's solution had been added, were acidified with 20% perchloric acid. Following freezing and boiling for 5 min, both supernatant and pellet were centrifuged at 17,000 × *g* for 5 min to remove denatured protein, and histamine was assayed fluorometrically (Theoharides *et al.*, 1982). The results are expressed as (supernatant/supernatant + pellet) × 100. The data for each incubation time and condition are expressed as mean ± standard error of the mean (s.e.mean) and statistical analysis was performed using ANOVA.

### *Intracellular calcium measurement*

Mast cells (10<sup>6</sup> ml<sup>-1</sup>) were incubated with 5 μg/ml of crimson calcium AM cell permeant probe (Cat. No. C-3018, Molecular Probes, Eugene, OR, U.S.A.) for 5 min at 37°C in Locke's solution without calcium and 0.1 mM EDTA. This indicator dye has been used effectively to measure intracellular calcium ion levels (Scheenen *et al.*, 1998; Herman, 1997; June & Rabinovitch, 1994). Mast cells were washed and resuspended in Locke's solution without calcium and 0.1 mM EDTA. Mast cells (180 μl) were then incubated with 0.1 μg ml<sup>-1</sup> compound 48/80 with or without preincubation with chondroitin sulphate for 10 min at room temperature in an open topped Sykes-Moore chamber. They were examined with a Confocal Laser Scanning Imaging System Odyssey XL (Norans Instruments) equipped with a Crypton-Argon laser set at 560λ (42% intensity) and attached to a Silicon Graphics computer. For image analysis, a field of cells (usually six to 10) was selected. Photography was started taking one frame every 20 s immediately after the addition of the study drug or the secretagogue (20 μl) for 10 min. Image analysis was performed using Image Pro Plus imaging software (Media Cybernetics, 8484 Georgia Avenue, Silver Springs, MD, U.S.A.). Each cell was analysed separately. Final magnification of 600× was used. Fifteen photographic

images for each sample were printed on one  $8 \times 10$  sheet of photographic paper and the images were subsequently digitized for analysis. First, a low threshold was established that extinguished the last image pixel. Thereafter, and at time points through 10 min, pixel values were recorded in response to drug treatment. The illuminated pixel values, representing areas, were then read from the program and plotted on graph paper.

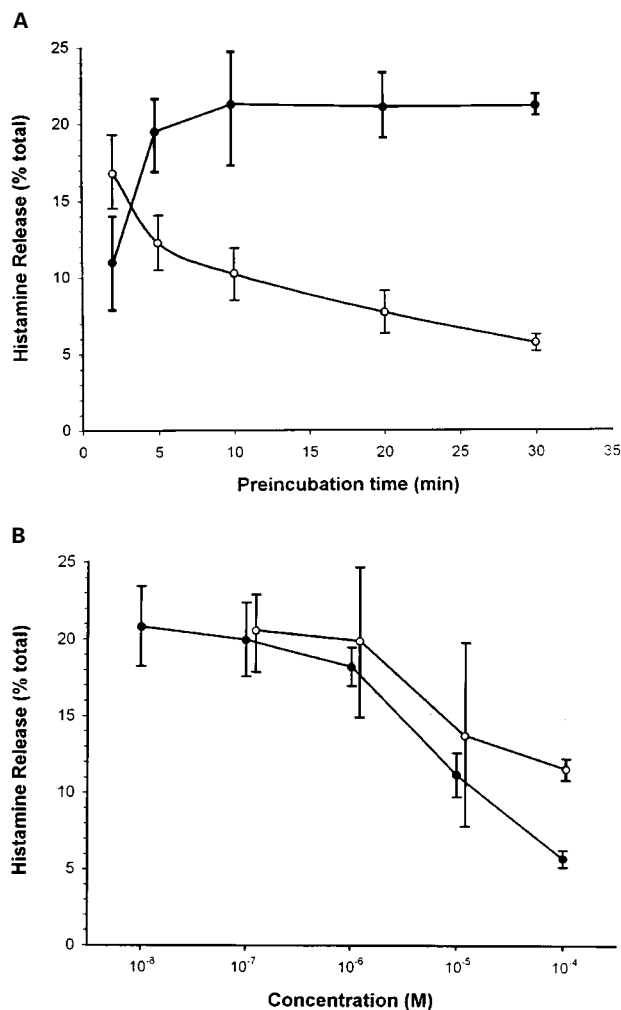
### Microscopy

For light microscopy, the cells were fixed with 4% paraformaldehyde in PBS for 30 min at room temperature. The cells were then washed with PBS, aliquoted onto slides and stored at  $-20^{\circ}\text{C}$  for further analysis. For electron microscopy, after immersion fixation of the cell pellet in 2.5% glutaraldehyde, the samples were washed in PBS, post-fixed in 2% osmium tetroxide, dehydrated in graded ethanol solutions and embedded in Epon. Semi-thin sections ( $0.5 \mu\text{m}$ ) were stained with toluidine blue, while ultrathin sections (1000 Å) were contrasted in 7.5% uranyl acetate in 50% ethanol and 0.1% aqueous lead citrate. Grids with the sections were examined and photographed using a Phillips CM-10 transmission electron microscope. For autoradiography, chondroitin sulphate was custom-tritiated at New England Nuclear (Boston, MA, U.S.A.) and was made available at specific activity of  $4.3 \text{ mCi ml}^{-1}$ ;  $10^5$  mast cells were incubated with  $2.5 \text{ mCi } ^3\text{H}$ -chondroitin sulphate for 30 min at  $37^{\circ}\text{C}$ . The cells were then washed twice, fixed and processed for autoradiography at the ultrastructural level without the uranyl acetate step; they were stained with lead citrate for 7.5 min (instead of 3 min when uranyl acetate is present). The cells were then examined and photographed using a Phillips-CM-10 transmission electron microscope; exposure was carried out for 7 days at  $4^{\circ}\text{C}$ .

## Results

Histamine release from rat CTMC stimulated with  $0.1 \mu\text{g ml}^{-1}$  of the classic mast cell secretagogue compound 48/80 (48/80) was  $22.1 \pm 1.3\%$  ( $n=19$ ). Preincubation with chondroitin sulphate resulted in inhibition of histamine release in response to 48/80 ( $0.1 \mu\text{g ml}^{-1}$  used for 30 min at  $37^{\circ}\text{C}$ ); inhibition increased with time and reached a maximum of 76.5% ( $P=0.0004$ ) by 10 min (Figure 1A). In contrast, the inhibitory action of cromolyn decreased rapidly if preincubation lasted for more than 1 min (Figure 1A). We then investigated whether this inhibitory effect may have been due to some electrostatic interaction between the negatively charged chondroitin sulphate and the cationic 48/80, leading to neutralization of the latter. Following incubation with  $10^{-5} \text{ M}$  chondroitin sulphate for 10 min at  $37^{\circ}\text{C}$ , mast cells were washed up to five times before stimulation with  $0.1 \mu\text{g ml}^{-1}$  48/80; the extent of inhibition was not diminished by the washes (results not shown). We also investigated how long the inhibitory activity of chondroitin sulphate lasted after two washes. Mast cells were incubated with  $10^{-5} \text{ M}$  chondroitin sulphate for 10 min at  $37^{\circ}\text{C}$ , washed twice in Locke's solution and then stimulated by  $0.1 \mu\text{g ml}^{-1}$  48/80 either immediately after or at 5, 15, 30, 60 and 120 min after the wash; there was no statistically significant reduction of the inhibitory effect even when 48/80 was added 2 h later (results not shown).

Pre-incubation with chondroitin sulphate for 10 min at  $37^{\circ}\text{C}$  inhibited histamine release in a dose-dependent manner



**Figure 1** (A) Time-course relationship of the inhibitory effect of chondroitin sulphate on compound 48/80-induced histamine release. Purified rat peritoneal mast cells ( $10^5 \text{ ml}^{-1}$ ) were preincubated with  $10^{-4} \text{ M}$  chondroitin sulphate or cromolyn for the times indicated followed by stimulation with  $0.1 \mu\text{g ml}^{-1}$  48/80 for 30 min at  $37^{\circ}\text{C}$ . Open circles = cromolyn; solid circles = chondroitin sulphate. (B) Dose-response relationship of the inhibitory effect of chondroitin sulphate on compound 48/80-induced histamine release. Purified rat peritoneal mast cells ( $10^5 \text{ ml}^{-1}$ ) were first incubated with or without chondroitin sulphate or cromolyn obtained from Sigma (St. Louis) for 10 or 1 min, respectively at  $37^{\circ}\text{C}$ . After incubation with the designated concentrations of chondroitin sulphate, mast cells were washed twice in Locke's solution and were stimulated with  $0.1 \mu\text{g ml}^{-1}$  48/80 for 30 min at  $37^{\circ}\text{C}$ . Open circles = cromolyn; solid circles = chondroitin sulphate.

(Figure 1B). The inhibitory effect of chondroitin sulphate was comparable to that of cromolyn, but chondroitin sulphate was more potent at the highest concentrations. Maximal inhibition of 76% ( $P=0.0004$ ) was seen at  $10^{-4} \text{ M}$  when histamine release decreased from  $22.0 \pm 1.3\%$  ( $n=19$ ) to  $5.7 \pm 0.6\%$  ( $n=13$ ). At  $10^{-5} \text{ M}$  ( $n=6$ ), histamine decreased to  $11.2 \pm 1.4\%$  (49.4% inhibition,  $P<0.05$ ) and at  $10^{-6} \text{ M}$  ( $n=6$ ), it decreased to  $18.2 \pm 1.3\%$  (17.3% inhibition,  $P<0.05$ ). There was only 9.4% inhibition at  $10^{-7} \text{ M}$  ( $n=7$ ) with histamine release at  $19.9 \pm 2.4\%$  which was borderline significant. At  $10^{-8} \text{ M}$  ( $n=4$ ), there was no inhibition and histamine release was  $20.8 \pm 2.6\%$  ( $P>0.05$ ).

The inhibitory effect of chondroitin sulphate extended to stimulation of rat CTMC by the neuropeptide SP, as well as by immunoglobulin E and anti-IgE; this inhibition also showed a dose-response relationship (Table 1). The inhibition seen with  $10^{-4} \text{ M}$  chondroitin sulphate on histamine release

triggered by  $5 \times 10^{-5}$  M SP was less (31.3%) than that seen with 48/80 (76%); this difference may possibly be due to the fact that stimulation by SP was stronger ( $43.1 \pm 11.9$ ,  $n=5$ ) than that induced by 48/80 ( $22.1 \pm 1.3$ ,  $n=19$ ) in the absence of extracellular calcium. When mast cells were stimulated by a lower concentration ( $10^{-5}$  M) of SP instead, histamine release was decreased to  $25.3 \pm 1.4\%$  ( $n=3$ ) and inhibition by  $10^{-4}$  M chondroitin sulphate increased to 69.2% ( $n=3$ ). Histamine secretion induced immunologically was  $19.5 \pm 2.3\%$  ( $n=4$ ) and was reduced to  $12.7 \pm 1.5\%$  ( $n=4$ ) by  $10^{-4}$  M chondroitin sulphate, an effect that was statistically significant ( $P=0.014$ ). Lower concentrations of chondroitin sulphate gave weaker and variable results.

In order to study the size or charge requirement of chondroitin sulphate, we compared its inhibitory action at  $10^{-5}$  M on immunologic stimulation of rat CTMC to that of high (HMW) and low molecular weight (LMW) heparin, as well as chondroitin sulphate disaccharide, and the monosaccharides D-glucosamine sulphate and N-acetyl-glucosamine ( $10^{-4}$  M). They were all weaker inhibitors than chondroitin sulphate (Table 2). For instance, chondroitin sulphate disaccharide inhibited immunologically stimulated histamine release by 23.6%, as compared to equimolar concentrations of the parent molecule (Table 2). The monosaccharides D-glucosamine sulphate and N-acetylglucosamine inhibited histamine release by 20.0 and 24.6%, respectively. Finally, HMW heparin was weaker than chondroitin sulphate with 25.5% inhibition, while LMW heparin inhibited histamine release only by 10.3% (Figure 2). Thus, it appeared that the size and degree of sulphation are important.

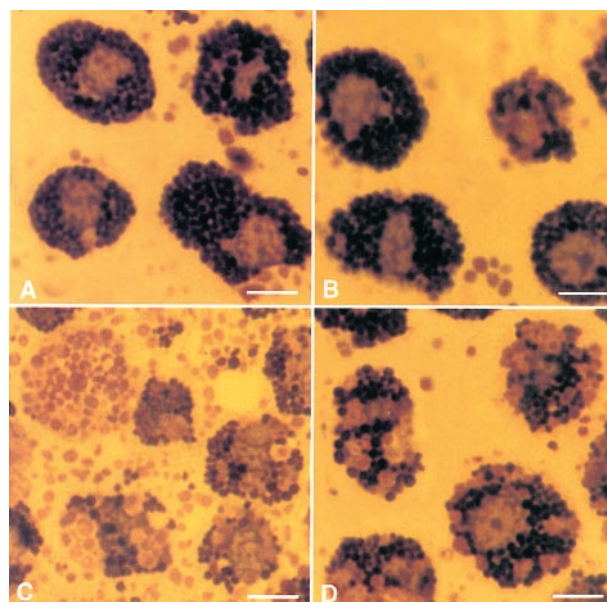
We also examined the morphologic appearance of rat CTMC treated with chondroitin sulphate. Control (untreated) mast cells and those preincubated with  $10^{-4}$  M chondroitin sulphate for 10 min at  $37^\circ\text{C}$  were intact (Figure 2A,B), as shown after staining with Giemsa. Stimulation with  $0.1 \mu\text{g ml}^{-1}$  48/80 made most mast cells release their contents to various degrees (compare Figure 2A,C). Preincubation with  $10^{-4}$  M chondroitin sulphate for 10 min at  $37^\circ\text{C}$ , before stimulation with 48/80, largely inhibited degranulation (compare Figure 2C,D). The possibility that the mechanism of action of chondroitin sulphate may be mediated through an effect on the availability of intracellular calcium ions was then investigated. Mast cells were loaded with the calcium indicator dye crimson red in the absence of extracellular calcium, were washed and observed in Locke's solution using a confocal microscope. Stimulation of secretion by  $0.1 \mu\text{g ml}^{-1}$  48/80 at room temperature led to a gradual increase in intracellular calcium ion levels shown in 20 s frames over a 10 min period (Figure 3A). Preincubation with  $10^{-4}$  M chondroitin sulphate for 10 min at  $37^\circ\text{C}$  prior to stimulation with 48/80 at room temperature inhibited calcium ion levels significantly (compare Figure 3A,B). The extent of

the inhibitory effect on intracellular calcium ion levels was confirmed with image analysis. Preincubation with chondroitin sulphate ( $10^{-4}$  M) decreased the number of pixels from  $1376.7 \pm 28.1$  ( $n=9$ ) illuminated 10 min after addition of

**Table 2** Effect of oligosaccharides on rat mast cell secretion triggered by IgE/Anti-IgE

Molecule <sup>a</sup>	Histamine release	% Inhibition
Control	$4.2 \pm 0.2$ ( $n=25$ )	
IgE/Anti-IgE	$19.5 \pm 2.3$ ( $n=4$ )	
Chondroitin sulphate	$2.9 \pm 0.2$ ( $n=14$ )	
Chondroitin sulphate + IgE	$12.7 \pm 1.5$ ( $n=4$ )	34.9*
ChS disaccharide	$4.5 \pm 2.0$ ( $n=9$ )	
ChS disaccharide + IgE	$14.9 \pm 4.9$ ( $n=9$ )	23.6*
D-Glucosamine sulphate	$4.1 \pm 0.1$ ( $n=3$ )	
D-Glucosamine sulphate + IgE	$15.6 \pm 0.5$ ( $n=3$ )	20.0*
N-acetyl-glucosamine	$3.2 \pm 0.2$ ( $n=3$ )	
N-acetyl-glycosamine + IgE	$14.7 \pm 1.1$ ( $n=3$ )	24.6*
Heparin (HMW)	$2.8 \pm 0.8$ ( $n=3$ )	
Heparin (HMW) + IgE	$14.3 \pm 0.3$ ( $n=3$ )	25.5*
Heparin (LMW)	$2.6 \pm 0.6$ ( $n=3$ )	
Heparin (LMW) + IgE	$17.5 \pm 0.6$ ( $n=3$ )	10.3*

<sup>a</sup>All at  $10^{-5}$  M; \* $P < 0.05$



**Figure 2** Photomicrographs of mast cells showing inhibition of degranulation by chondroitin sulphate. Purified mast cells (>95%) were washed and resuspended in the Locke's solution. They were observed and photographed after staining with Giemsa. (A) Control; (B) preincubated with  $10^{-4}$  M chondroitin sulphate; (C) stimulated with  $0.1 \mu\text{g ml}^{-1}$  C48/80 alone for 30 min at  $37^\circ\text{C}$  or (D) after 10 min preincubation with  $10^{-4}$  M chondroitin sulphate. Bar = 10 microns.

**Table 1** Effect of chondroitin sulphate on mast cell secretion induced by different stimuli

Conditions	Control	Histamine release (% total) SP <sup>a</sup>	IgE/anti-IgE
Buffer	$4.2 \pm 0.2$ ( $n=25$ )	$43.1 \pm 11.9$ ( $n=5$ )	$19.5 \pm 0.3$ ( $n=4$ )
Heparin ( $10^{-4}$ M)	$2.8 \pm 0.8$ ( $n=3$ )	$35.5 \pm 1.5$ (17.6%↓)	$14.3 \pm 0.3$ (25.5%↓)
ChS ( $10^{-4}$ M)	$2.9 \pm 0.2$ ( $n=14$ )	$29.6 \pm 8.3$ ( $n=5$ )#	$12.7 \pm 1.5$ ( $n=4$ )#
ChS ( $10^{-5}$ M)	$3.1 \pm 0.8$ ( $n=6$ )	$34.7 \pm 6.8$ ( $n=3$ )	$15.1 \pm 2.1$ ( $n=3$ )
ChS ( $10^{-6}$ M)	$3.4 \pm 0.6$ ( $n=6$ )	$39.3 \pm 9.2$ ( $n=3$ )	$17.1 \pm 2.3$ ( $n=3$ )
Maximal inhibition (%)		31.3*	34.9*

ChS = chondroitin sulphate;  $n$  = no. of experiments; <sup>a</sup> $5 \times 10^{-5}$  M; \* $P < 0.05$ .

compound 48/80 at 24°C to  $399.4 \pm 51.0$  ( $n=9$ ), more than a 70% ( $P<0.05$ ) reduction (Table 3).

The inhibitory effect of chondroitin sulphate was also confirmed by transmission electron microscopy. Ultrastructural observations showed most control mast cells (Figure 4A) and those treated with  $10^{-4}$  M chondroitin sulphate for 10 min at 37°C (Figure 4B) to contain intact, homogenous electron dense granules. In contrast, mast cells stimulated by  $0.1 \mu\text{g ml}^{-1}$  48/80 had undergone extensive degranulation and had lost the electron dense content of most of their secretory granules (Figure 4C). Mast cells incubated with  $10^{-4}$  M chondroitin sulphate for 10 min at 37°C before stimulation with  $0.1 \mu\text{g ml}^{-1}$  48/80 also contained mostly intact electron dense granules (Figure 4D). The possible site of action of chondroitin sulphate was investigated by using tritiated chondroitin sulphate. Autoradiography at the ultrastructural level showed that, after a 30 min incubation,

$^3\text{H}$ -chondroitin sulphate radioactivity could not be removed even by five washes and was mostly associated with the mast cell surface and perigranular membranes (Figures 5 and 6). It is interesting to note that  $^3\text{H}$ -chondroitin sulphate-related radioactivity appeared to be least associated with the nucleus or secretory granules that had undergone exocytosis (Figures 5B and 6).

We then investigated if the inhibitory effect of chondroitin sulphate was limited only to rat CTMC. For that reason, we tested it against immunologic stimulation of RBL and human cultured mast cells. While chondroitin sulphate ( $10^{-5}$  M) inhibited immunologic histamine release from rat CTMC by 22% (Table 4), it had no effect (9.4% inhibition,  $P>0.05$ ) on histamine release from RBL cells which are homologous to rat MMC (Table 4). Histamine release from umbilical cord-derived human mast cells was decreased (Table 4) from  $24.7 \pm 5.8\%$  to  $21.8 \pm 3.7\%$ , a 12.2% reduction ( $P<0.05$ ). Cromolyn used under the same conditions inhibited CTMC by 16.4%, had no effect on RBL cells, and inhibited human cultured mast cells by 4.5% which was not statistically significant.

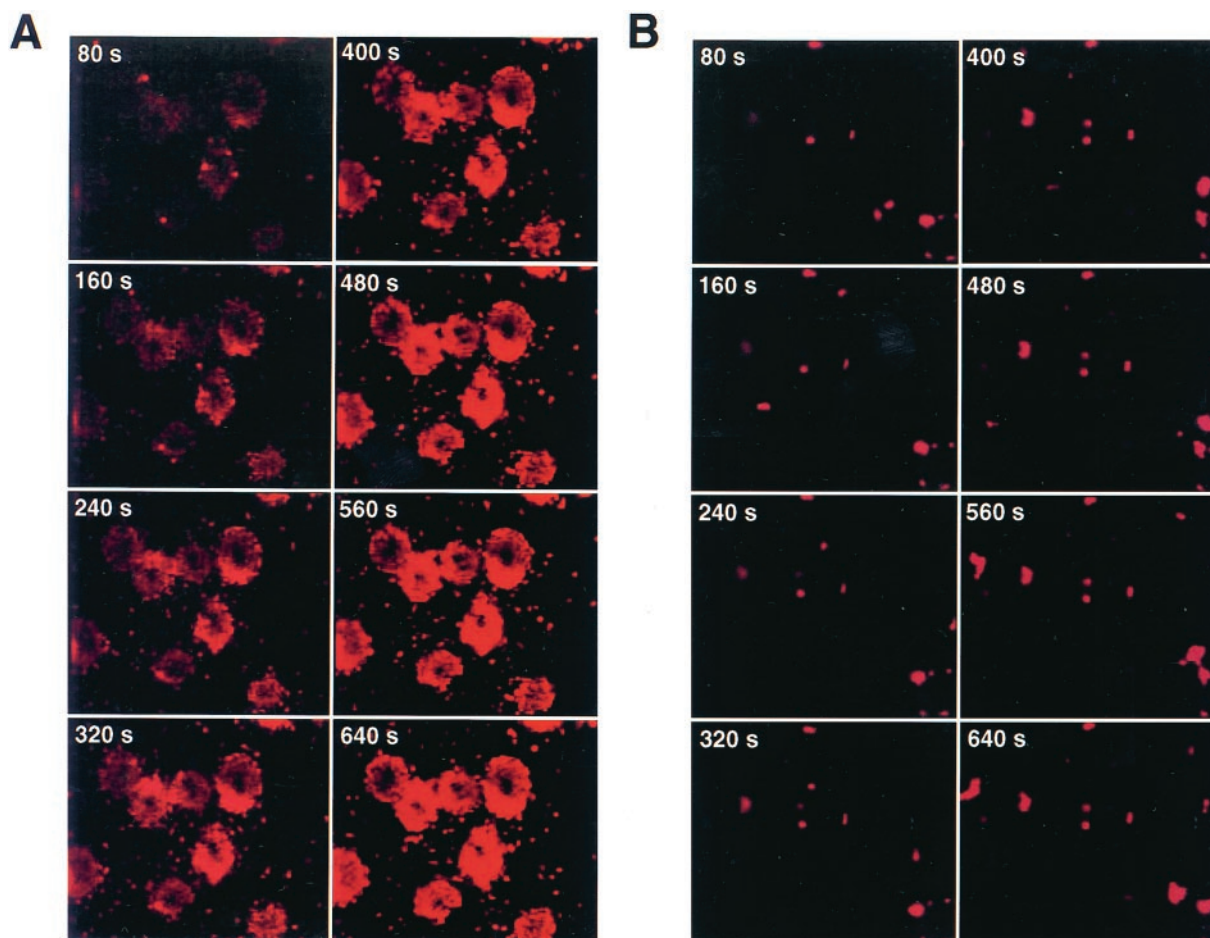
**Table 3** Effect of chondroitin sulphate on intracellular calcium ion levels

Conditions <sup>a</sup>	No. of pixels illuminated
48/80	$1376.7 \pm 28.1$ ( $n=9$ )
ChS + 48/80	$399.4 \pm 51.0^*$ ( $n=9$ )

<sup>a</sup>With or without preincubation with  $10^{-4}$  M chondroitin sulphate for 10 min at 37°C and then evaluated (for 60 s) at 10 min after addition of  $0.1 \mu\text{g ml}^{-1}$  48/80 at room temperature;  $n$  = number of cells evaluated. \* $P<0.001$

## Discussion

The present results show that chondroitin sulphate is a potent inhibitor of mast cell secretion. Chondroitin sulphate is a natural constituent of connective tissues (Hardingham, 1986), especially cartilage (Lindahl & Hook, 1978). Chondroitin



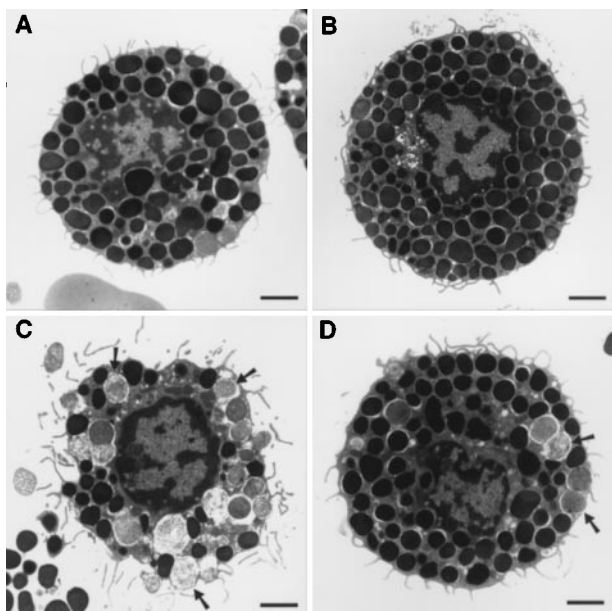
**Figure 3** Photomicrographs of mast cells obtained every 20 s showing the effect of chondroitin sulphate on intracellular calcium ion levels. Mast cells were observed after loading with Crimson calcium AM. They were either (A) stimulated with  $0.1 \mu\text{g ml}^{-1}$  48/80 alone for 10 min at room temperature or (B) after 10 min preincubation with  $10^{-4}$  M chondroitin sulphate. Bar = 10 microns.

sulphate is also present in both rat MMC (Seldin *et al.*, 1985b; Razin *et al.*, 1983) and basophils (Orenstein *et al.*, 1978; Reilly *et al.*, 1988; Seldin *et al.*, 1985b; Metcalfe *et al.*, 1984), as well as in human CTMC (Krilis *et al.*, 1992) and umbilical cord-derived human mast cells (Blom *et al.*, 1996). The inhibitory action of chondroitin sulphate on histamine release was more potent than of the 'antiallergic' drug cromolyn, especially at higher concentrations. Heparin, the structure of which is quite similar to that of chondroitin

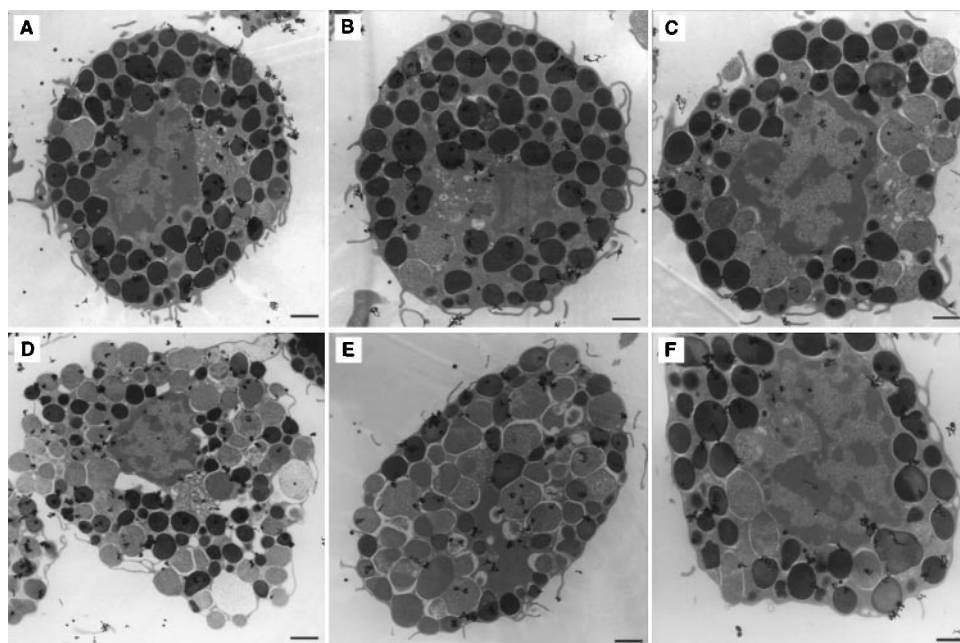
sulphate and is also stored in mast cells, had been shown to inhibit *in vivo* release of histamine (Dragstedt *et al.*, 1942), the immediate response to antigen in the skin and lungs of allergic subjects (Bowler *et al.*, 1993), as well as bronchoconstriction in exercise-induced asthma (Ahmed *et al.*, 1993a). Heparin was also shown to prevent allergic airway hyperresponsiveness (Ahmed *et al.*, 1994) and to have a protective effect on immunologic and non-immunologic tracheal smooth muscle contraction *in vitro* (Abraham *et al.*, 1996; Ahmed *et al.*, 1992). In fact, just as cromolyn and chondroitin sulphate appear to have roughly equivalent inhibitory activity on CTMC secretion, heparin and the cromolyn analogue nedocromil both induced inhibition of about 50% (Abraham *et al.*, 1996) on tracheal smooth muscle contraction.

While the inhibition by cromolyn disappeared with preincubation times of more than 1 min, the inhibitory action of chondroitin sulphate started at 1 min and progressively increased over 30 min. It was previously shown that the inhibitory action of intravenous heparin in the immediate cutaneous reaction in allergic sheep was enhanced by 60% when the duration of pretreatment was increased from 20 to 60 min (Ahmed *et al.*, 1993b). Like our findings showing that chondroitin sulphate inhibits both 48/80-induced and immunologic stimulation of mast cells, heparin had been shown to inhibit similar responses in isolated human uterine mast cells (Lucio *et al.*, 1992). It was somewhat surprising that the inhibitory effect of chondroitin sulphate was more potent for histamine release induced by 48/80 than with SP, since both are thought to occur through the same mechanism (Mousli *et al.*, 1990). One possible explanation may be the fact that in the absence of extracellular calcium ions, the effect of 48/80 is much weaker than that of SP (Erjavec *et al.*, 1981), suggesting that the stronger the response the weaker the inhibition; our preliminary results using less SP appear to support this explanation.

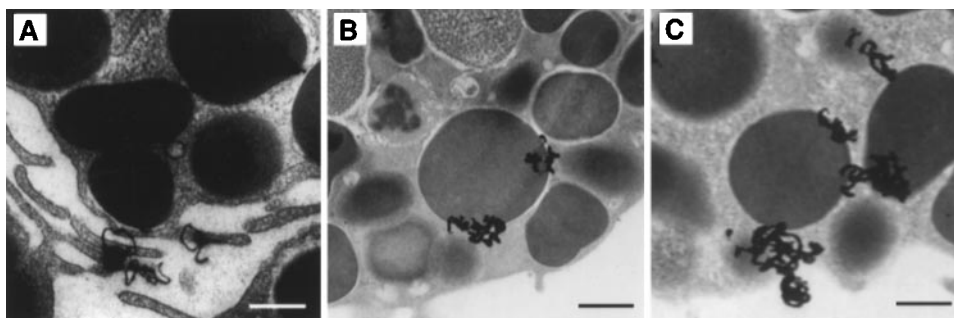
Our ultrastructural observations showed that chondroitin sulphate prevented mast cell degranulation. Moreover, auto-



**Figure 4** Electron photomicrographs of mast cells showing the inhibitory effect of chondroitin sulphate. (A) Control; (B)  $10^{-4}$  M chondroitin sulphate for 30 min at  $37^{\circ}\text{C}$ ; (C)  $0.1 \mu\text{g ml}^{-1}$  compound 48/80 for 30 min at  $37^{\circ}\text{C}$ ; (D) preincubation with  $10^{-4}$  M chondroitin sulphate for 30 min at  $37^{\circ}\text{C}$ , washed twice, and stimulated with  $0.1 \mu\text{g ml}^{-1}$  compound 48/80 for 30 min at  $37^{\circ}\text{C}$ . Bar = 1 micron.



**Figure 5** Electron autoradiographs showing non-random distribution of  $^3\text{H}$ -chondroitin sulphate after 30 min incubation at  $37^{\circ}\text{C}$ . (A–C) Note deposition of  $^3\text{H}$ -chondroitin sulphate-related radioactivity (grains) mostly in association with intact secretory granules and the cell surface; (D–F) note relative absence of grains from the nucleus and secretory granules that had undergone exocytosis. Magnification =  $\times 15,600$ . Bar = 1 micron.



**Figure 6** Electron autoradiographs showing the presence of  $^3\text{H}$ -chondroitin sulphate after 30 min of incubation at  $37^\circ\text{C}$ . (A) Note deposition of  $^3\text{H}$ -chondroitin sulphate-related radioactivity (grains) in association with the mast cell surface, especially filopodia. (B,C) Note grains are adjacent to secretory granule membranes. Magnification =  $\times 19,800$ . Bar = 0.5 microns.

**Table 4** Effect of chondroitin sulphate on immunologic stimulation of mast cells obtained from different sources

Conditions <sup>a</sup>	Rat peritoneal	Histamine release (% total)	
		RBL	Human umbilical
Spontaneous	4.2 $\pm$ 0.2 (n = 25)	0.8 $\pm$ 0.4 (n = 5)	10.7 $\pm$ 0.7 (n = 4)
ChS ( $10^{-5}$ M)	3.1 $\pm$ 0.8 (n = 6)	0.6 $\pm$ 0.2 (n = 3)	14.7 $\pm$ 1.2 (n = 4)*
Cromolyn ( $10^{-5}$ M)	3.7 $\pm$ 0.3 (n = 5)	0.9 $\pm$ 0.4 (n = 3)	9.3 $\pm$ 1.7 (n = 3)
IgE/anti-IgE	19.5 $\pm$ 2.3 (n = 4)	9.6 $\pm$ 2.8 (n = 4)	24.7 $\pm$ 5.8 (n = 4)
ChS ( $10^{-5}$ M) + IgE	15.1 $\pm$ 2.1 (n = 4)	8.7 $\pm$ 4.0 (n = 4)	21.8 $\pm$ 3.7 (n = 4)
Inhibition by ChS (%)	22.3*	9.4	12.2*
Cromolyn ( $10^{-5}$ M) + IgE	16.3 $\pm$ 1.8 (n = 5)	0.8 $\pm$ 0.2 (n = 3)	23.6 $\pm$ 2.1 (n = 3)
Inhibition by cromolyn	16.4*	–	4.5

ChS = chondroitin sulphate; n = no. of experiments; <sup>a</sup>All drugs were added 1 min prior to stimulation; \* $P < 0.05$ .

radiographic images suggest that chondroitin sulphate is mostly associated with the mast cell surface and the secretory granule membranes, while largely sparing the nucleus and granules that had undergone exocytosis. Similar findings had been reported in studies of  $^{35}\text{S}$ -chondroitin sulphate uptake in other cultured cells where chondroitin sulphate first interacted with the cell surface and by 2 h was found mostly intracellularly (Saito & Uzman, 1971c). Our results showed that the plasma membrane and the extruded secretory granules are preferentially fluorescent in our confocal microscopic images. This is in part due to the fact that the confocal microscope focuses on the surface of live cells. Even with conventional microscopy, however, the plasma membrane of cells in their resting state is preferentially stained for calcium ions (Tasaka *et al.*, 1986; Monck *et al.*, 1992). Previous reports using Quin-2 or Fura-2 (Penner *et al.*, 1988; Tasaka *et al.*, 1986; Almers & Neher, 1985; Monck *et al.*, 1992) also showed that during stimulation of secretion, there is an increase in calcium ions associated with extruded secretory granules, possibly due to indicator dye trapped inside the granules fluorescing upon binding extracellular calcium (Almers & Neher, 1985). There is also binding of the cationic extracellular calcium to the negatively charged extruded granule proteoglycans that permits the intense granule-associated fluorescence. Real time sequence of 20 s frames showed the gradual increase of intracellular calcium upon stimulation of mast cells with 48/80 and its inhibition by chondroitin sulphate. In these frames, not only the time-dependent increase is appreciated, but the cells act as their own controls before stimulation. The precise mechanism through which chondroitin sulphate inhibits mast cell secretion is not entirely clear. Our results indicate the interaction of chondroitin sulphate with mast cells somehow led to inhibition of intracellular free calcium ion levels. This action could not be through a calcium ion channel as 48/80 is known to induce secretion utilizing intracellular calcium (Theoharides *et al.*, 1982) and SP stimulates mast cells in the absence of

extracellular calcium (Erjavec *et al.*, 1981). Heparin had previously been reported to inhibit inositol triphosphate-induced intracellular calcium release from permeabilized rat liver cells (Hill *et al.*, 1987).

We also do not know whether the inhibitory effect of chondroitin sulphate is mediated through its surface binding and/or some action on the perigranular membrane or both. However, it is interesting to note that mast cell treatment with neuraminidase prevented histamine release induced by SP (Cocchiara *et al.*, 1997), implying that incorporation of chondroitin sulphate in the plasma membrane may interfere with activation, especially by neuropeptides. How chondroitin sulphate gets across the cell membrane can only be theorized at the present, but a number of publications have shown internalization of both chondroitin sulphate (Saito & Uzman, 1971a,b,c) and heparin (Letourneur *et al.*, 1995a,b) by active uptake through receptor-mediated endocytosis.

Proteoglycans liberated from activated mast cells and/or connective tissues could act as natural inhibitors of mast cell secretion, thus also reducing the extent of local inflammation. The mechanism of inhibitory action of chondroitin sulphate may be through two different mechanisms. Firstly, competition of compound 48/80 or SP binding to surface proteoglycans through membrane incorporation so that it can not be washed off; for instance, neuraminidase-treated rat peritoneal mast cells could not be stimulated by 48/80 or SP (Mousli *et al.*, 1989) and lectins specific for N-acetyl galactosamine and N-acetyl glucosamine oligomers blocked stimulation by 48/80 (Matsuda *et al.*, 1994). Secondly, chondroitin sulphate may interfere with the action of inositol triphosphate and subsequent increases in intracellular calcium ions, especially since heparin has been shown to act as a competitive inhibitor of inositol triphosphate receptors (Kobayashi *et al.*, 1988; Ahmed *et al.*, 1994).

Chondroitin sulphate may have additional actions leading to inhibition of inflammatory responses *in vivo*. Since proteoglycans have been shown to extend the life of growth

factors (Brittis *et al.*, 1992; Nurcombe *et al.*, 1993; Kan *et al.*, 1993), exogenously provided chondroitin sulphate may act as a decoy to prevent or limit the action of growth factors on local immune cells or neurons. The possibility that any growth or other factors bound to chondroitin sulphate may have contributed to the inhibitory action of chondroitin sulphate in our experiments is rendered unlikely due to the fact that in certain experiments chondroitin sulphate was first dissolved in high salt to disrupt any loose interactions; it was then filtered through a 50 K exclusion filter which would have allowed any smaller molecules to be washed through. An additional possible beneficial effect of chondroitin sulphate in reducing inflammation would be its ability to bind intercellular adhesion molecules (Nelson *et al.*, 1993), often expressed in response to TNF- $\alpha$  released from mast cells (Gordon *et al.*, 1990; Walsh *et al.*, 1991), and to neutralize proteolytic enzymes released during inflammatory responses (Zehnder & Galli, 1999; Humphries *et al.*, 1999; Forsberg *et al.*, 1999). It is interesting to note that heparin sulphate was recently shown to bind to CD48 (Ianelli *et al.*, 1998), a molecule that is upregulated in various autoimmune disorders. Such findings are provocative in view of the fact that mast cells are increasingly implicated in inflammatory processes (Galli, 1993) especially those exacerbated by stress (Theoharides, 1996). For instance, mast cells have been implicated in sterile inflammatory disorders such as asthma (Barnes, 1988; Lewis & Austen, 1977), irritable bowel syndrome (Weston *et al.*, 1993) and interstitial cystitis in the urinary bladder (Theoharides *et al.*, 1998), in all of which a deficit of glycosaminoglycans has been reported (Shute *et al.*, 1997; Hurst *et al.*, 1996; Burton & Anderson, 1983).

A number of non-mast cell natural or synthetic compounds have been reported to inhibit mast cell secretion. These include lodoxamides (Johnson, 1980), doxantrozole (Pearce *et al.*, 1982; Fox *et al.*, 1988), quercetin (Fewtrell & Gomperts, 1977; Fox *et al.*, 1988) and certain antihistamines such as hydroxyzine (Theoharides *et al.*, 1985; Fischer *et al.*, 1995) and ketotifen (Nemeth *et al.*, 1987). Glucocorticoids do not affect anaphylactic mast cell degranulation, but prolonged treatment inhibits the synthesis of arachidonic acid products such as prostaglandins, thromboxanes and leukotrienes in response to immunologic stimulation (Robin *et al.*, 1985; Marquardt & Wasserman, 1983). On the contrary, even overnight steroid treatment of mast cells did not affect either histamine or arachidonic acid release induced by 48/80 or somatostatin (Heiman & Crews, 1984). Cromolyn and its structurally related nedocromil known as 'mast cell stabilizers' (Theoharides *et al.*, 1980), are available for allergic conjunctivitis, rhinitis, asthma and food allergies (Enerbäck & Bergström, 1989; Theoharides *et al.*, 1980; Okayama *et al.*, 1992), even though they do not appear to be able to inhibit human mast cells (Okayama *et al.*, 1992), as also shown herein. Moreover, their inhibitory action on histamine release is only modest, short-lived and does not inhibit histamine secretion from MMC (Barrett & Metcalfe, 1985; Pearce *et al.*, 1982; Fox *et al.*, 1988). Nevertheless, cromolyn was shown to inhibit tumour necrosis- $\alpha$  (TNF- $\alpha$ ) release from both CTMC and MMC, an action that required 2 h pretreatment (Bissonnette *et al.*, 1995). It is interesting that low molecular weight (LMW) heparin preferentially inhibited TNF- $\alpha$  and IL-4 production without affecting degranulation (Baram *et al.*, 1997). Our preliminary results show that LMW heparin, as well as chondroitin sulphate disaccharide and related monosaccharides are weaker inhibitors of histamine secretion. Weak inhibition on mast

cell secretion, has previously been reported for other mast cell derived molecules (Theoharides, 1990) such as lipoxins (Conti *et al.*, 1992), histamine through H-3 receptors in the brain (Rozniecki *et al.*, 1999) and interleukin-8 (Kuna *et al.*, 1991). Inhibition of mast cell production of nitric oxide (NO) enhanced histamine release in response to compound 48/80 and SP (Brooks *et al.*, 1999), but diminished mast cell dependent TNF- $\alpha$ -mediated cytotoxicity (Bissonnette *et al.*, 1991). Both the constitutive and the antigen-induced NO production was inhibited by IL-10; however, long-term (24 h) incubation with IL-10 enhanced histamine release from CTMC (Lin & Befus, 1997). These findings imply that there may be differential inhibition of release of mast cell mediators we first reported for biogenic amines (Theoharides *et al.*, 1982). A number of subsequent reports confirmed our original observation. For instance, vasoactive amines were differentially released in T-cell-dependent, as compared to IgE-dependent hypersensitivity reactions (Van Loveren *et al.*, 1984). Differential release of histamine without prostaglandin D<sub>2</sub> (Benyon *et al.*, 1989) was reported by peptides (Levi-Schaffer & Shalit, 1989) and 48/80 (van Haaster *et al.*, 1995), which also released almost no leukotriene C4 (Benyon *et al.*, 1989). The anaphylatoxin C5a induced high histamine release without any leukotrienes from human basophils (Dahinden *et al.*, 1989). Interleukin-10 stimulated IL-6 and TNF- $\alpha$  production without affecting histamine release (Marshall *et al.*, 1996), while the phosphatidylinositol 3-kinase inhibitor Wortmannin inhibited mast cell exocytosis, but not IL-6 production (Marquardt *et al.*, 1996). Stimulation with SCF resulted in IL-6 release without histamine or leukotriene C4 (Gagari *et al.*, 1997) and bacterial lipopolysaccharide induced release of IL-6 without histamine (Leal-Berumen *et al.*, 1994).

Differential activation of mast cells may also vary due to their content, source, as well as experimental or pathological conditions. In addition to the well-known differences in human protease content (Schwartz, 1987), human mast cells also differ in their proteoglycan (Pipkorn *et al.*, 1988) and cytokine (Bradding *et al.*, 1995) content. The secretory responsiveness of mast cells also varies considerably. Human mast cells appear capable of constitutively releasing histamine without tryptase (Granerus *et al.*, 1994). MMC do not secrete in response to 48/80 or neuropeptides (Barrett & Metcalfe, 1985; Pearce *et al.*, 1982), even though they did respond weakly only to high concentrations of SP (Ali *et al.*, 1986; Shanahan *et al.*, 1985). Human mast cells are refractory to the action of 48/80 or polylysine (Lowman *et al.*, 1988b; Church *et al.*, 1982), while skin mast cells are responsive to these synthetic compounds, as well as to SP, somatostatin and vasoactive intestinal peptide (VIP) (Lowman *et al.*, 1988a,b; Piotrowski & Foreman, 1985).

### Conclusion

The present findings suggest that chondroitin sulphate may be useful as a natural 'anti-allergic' agent. In addition, evidence that mast cells are involved in neuroinflammatory processes (Church *et al.*, 1989; Foreman, 1987; Goetzl *et al.*, 1985; Stead & Bienenstock, 1990; Theoharides, 1996) and are present in inflamed joints (Crisp *et al.*, 1984; Wasserman, 1984; Metzler & Xu, 1997), may help explain (Theoharides & Boucher, 1999) a meta-analysis suggesting that chondroitin sulphate may be beneficial in osteoarthritis (McAlindon, *et al.*, 2000). In fact the US National Institutes of Health recently sponsored a trial (NIH-NIAMS-98-2) to test the efficacy of chondroitin sulphate in osteoarthritis.



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