Kurloff cell proteoglycans

Evidence for de novo synthesis of chondroitin sulphate proteoglycans by purified Kurloff cells

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This paper reports the first direct demonstration of de novo synthesis of chondroitin sulphate proteoglycans by Kurloff cells. This was achieved using highly purified splenic Kurloff cells labelled in vitro with [^{35}S]sulphate and D-[U-^{3}H]glucosamine. A single population of sulphated proteoglycans was observed after dissociative extraction, DEAE-cellulose chromatography, Sepharose CL 6B chromatography and fluorography after electrophoresis. These were large, highly anionic proteoglycans and were completely digested by chondroitinase AC or ABC. Moreover, glycosaminoglycan extracted from Kurloff cells had the electrophoretic mobility of control chondroitin sulphate.

(Kurloff cell, Spleen cell) *Leukocyte* *Proteoglycan* *Glycosaminoglycan* *Chondroitin sulfate*

1. INTRODUCTION

Kurloff cells (KC) [1] are found in the blood, spleen, thymus, lungs, liver, bone marrow and placenta of guinea pigs. They contain a large (11 μm diameter), periodic acid-Schiff positive inclusion body (KB) which has long been histochemically defined as a 'mucoprotein-mucopolysaccharide complex' [2]. Under the electron microscope, the KB appears as a dense, homogeneous and amorphous central matrix surrounded by myelinic figures and delineated by a single trilaminar membrane [3]. KC are more numerous in females than in males and increase considerably in number during pregnancy. Ledingham [4] showed that they were produced in both male and female guinea pigs after the administration of oestrogens. By extraction of KC-rich spleens (obtained from oestrogenized guinea pigs) a mucopolysaccharide fraction was characterized as chondroitin sulphate (CS) [5] and then as CS proteoglycan (PG) [6]. Spleens obtained from oestrogenized guinea pigs were shown to have a higher glycosaminoglycan (GAG) content than control spleens and this finding was related [7] to the dramatic increase in splenic KC numbers induced by oestrogens. Moreover, very little incorporation of [^{35}S]sulphate into the splenic GAG was observed [7,8]. The intracellular localization of radiolabelled sulphate in the KB was demonstrated by autohistoradiography in guinea pigs given this isotope [2,9,10]. PG analogous to Kurloff PG were extracted from the spleen of pregnant sheep, pigs, rats and humans [11]. As these species were devoid of KC, they may have a specialized line of lymphoid cells which are not histologically distinguishable, as KC in guinea pigs, but contain similar PG and have a similar function. The extraction of a ubiquitous extra- or intracellular PG (possibly dependent on plasma oestrogen levels)

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could not be excluded. The presence of CS-PG in the KB was very probable but the de novo synthesis of these PG by the KC itself has not yet been directly demonstrated [1,10].

In this study, GAG were extracted from 95% purified and viable splenic KC and characterized by electrophoresis, as a KC purification technique has been available since 1984 [12]. These purified KC were also incubated with radiolabelled sulphate or glucosamine and Kurloff CS-PG were then extracted and characterized.

2. MATERIALS AND METHODS

2.1. *Guinea pig oestrogenization and KC purification*

Oestrogenization of guinea pigs and KC purification were performed as previously described [12].

2.2. *Extraction and electrophoresis of Kurloff GAG*

KC or total spleens were homogenized in cold acetone; after centrifugation and drying, the pellets were digested, in turn, with pronase (1 mg/ml of 0.1 M Tris-HCl buffer, pH 7.5, 0.005 M $CaCl_2$) for 48 h at 50°C, ribonuclease and deoxyribonuclease (2 mg/ml of 0.005 M Tris-HCl buffer, pH 7.0, 0.012 M $MgSO_4$) for 20 h at 30°C. GAG were isolated and purified by repeated cetylpyridinium chloride and ethanol precipitations [13].

Electrophoresis of GAG on a cellulose acetate membrane was performed in 0.05 M LiCl, pH 2.0 [14]. The strips were transferred, without drying, to a 1% alcian blue solution in 2% acetic acid for 30 min. CS and hyaluronic acid were used as a control.

2.3. *Uronic acid and protein determination*

Uronic acid and protein were determined according to Bitter and Muir [15] and Lowry et al. [16], respectively.

2.4. *Metabolic labelling of KC and extraction of PG*

Incubation media were buffered with 1% of 1 M Hepes at pH 7.2 supplemented with antibiotics (penicillin, 200 IU/ml; streptomycin, 100 μg/ml; fungizone, 0.25 μg/ml) and 10% Ultroser HY (IBF). After 30 min prestarving, 200–300 $\times$ 10^6, 95% pure and 95% viable KC were incubated with either 300 μCi [^{35}S]sulphate (Amersham, spec. act. 25–40 Ci/mg) for 12–18 h in 5 ml Fischer medium [17] or 20 μCi D-[1-^{3}H]glucosamine (CEA, spec. act. 10–15 Ci/mM) for the same time in a glucose-free, Dulbecco phosphate buffered saline [18] supplemented with minimum Eagle medium [19], amino acid and vitamin solutions.

After incubation, KC were washed three times in Hank's balanced salt solution by successive spinning and resuspension. The last pellet was resuspended in 4 ml extraction buffer: 4 M guanidinium chloride, 0.05 M Tris-HCl, pH 6.8, containing 10 mM EDTA, 100 mM 6-aminohexanoic acid, 5 mM benzaminidium chloride, 10 mM ethylmaleimide, 0.36 mM pepstatin and 1 mM phenylmethanesulphone fluoride as protease inhibitors [20]. The suspension was stirred at 4°C for 20 h and centrifuged at 100000 $\times$ g for 60 min at 4°C.

2.5. *Separation of glycoconjugates*

2.5.1. DEAE-cellulose chromatography

The D-(1-^{3}H)- or ^{35}S-labelled supernatants were dialysed against two 400 ml batches of 7 M urea containing the cocktail of protease inhibitors. 2 ml of the urea soluble, dialysed sample was then applied to a DEAE-cellulose column (1.1 $\times$ 5 cm) equilibrated with 7 M urea in 0.05 M Tris-HCl buffer, pH 6.8, containing protease inhibitors. The column was eluted with the equilibration buffer (8 ml) and then with a 43 ml linear gradient of NaCl (0–1.2 M) at a flow rate of 25 ml/h. 1 ml fractions were collected and the radioactivity determined of 0.1 ml aliquots.

2.5.2. Sepharose column chromatography

The sulphated peak from DEAE-cellulose chromatography was dialysed extensively against distilled water. The non-diffusible material was freeze-dried, dissolved in 0.5 ml extraction buffer, layered on a Sepharose CL 6B column (0.7 $\times$ 42 cm) and eluted with the same dissociative buffer (flow rate, 3 ml/h). 0.2 ml fractions were collected and radioactivity was assayed.

2.6. *Digestion with chondroitinases*

The radiosulphated peak obtained after successive DEAE-cellulose and Sepharose CL 6B

chromatography was dialysed extensively against water and freeze-dried. Hydrolysis was performed with 0.2 IU chondroitinase ABC or AC (Sigma) in 0.05 M Tris/acetate buffer (pH 8.0) containing 0.05 M NaCl for 18 h at 37°C. After digestion, solid guanidinium chloride was added to the sample to give a final concentration of 4 M for Sepharose CL 6B chromatography.

Alternatively, aliquots of the sulphated peak obtained from DEAE-cellulose were directly subjected to chondroitinase treatment under the same conditions. Homologous digested and undigested samples were mixed with Laemmli's sample buffer for electrophoresis.

2.7. *Electrophoresis of PG and fluorography*

PAGE-10% SDS was performed according to Laemmli [21]. Fluorography [22] with Enlightning (NEN) was performed before exposure at −70°C for 30 days to Kodak X-Omat AR films.

3. RESULTS

3.1. *Extraction of GAG from purified KC and from whole spleen*

The GAG concentration, expressed in μg uronic acid/mg protein, was 4.87 in purified KC whereas it was only 1.75 in splenic extracts. These results suggest that a major part of the splenic GAG was located in KC. After electrophoresis of the GAG obtained from KC, only one alcian blue band was coloured on cellulose acetate. Its electrophoretic mobility was the same as control CS. No material was found with the electrophoretic mobility of hyaluronic acid.

3.2. *Separation of Kurloff PG by DEAE-cellulose*

Radiosulphated cellular extracts, corresponding to 150 × 10^6 KC, were chromatographed on DEAE-cellulose. Fig.1 shows a single peak of sulphated material that eluted at an NaCl concentration of 0.55 M. The peak fractions were pooled and the resulting sample was dialysed against distilled water containing protease inhibitors and further analyzed as described below (sections 3.3–3.5).

After incubation with D-[1-^{3}H]glucosamine, similar radiolabeled KC extracts were chromatographed under the same conditions on DEAE-cellulose (fig.2). Two peaks were eluted with the NaCl-free buffer. They contained almost all the protein of the sample and corresponded to glycoproteins [23], as demonstrated by PAGE-SDS followed by PAS staining (not shown). Three other peaks were eluted with NaCl concentrations of 0.18 M, 0.40 M and 0.55 M. The latter peak was eluted at exactly the same NaCl concentration as the single radiosulphated peak (fig.1).

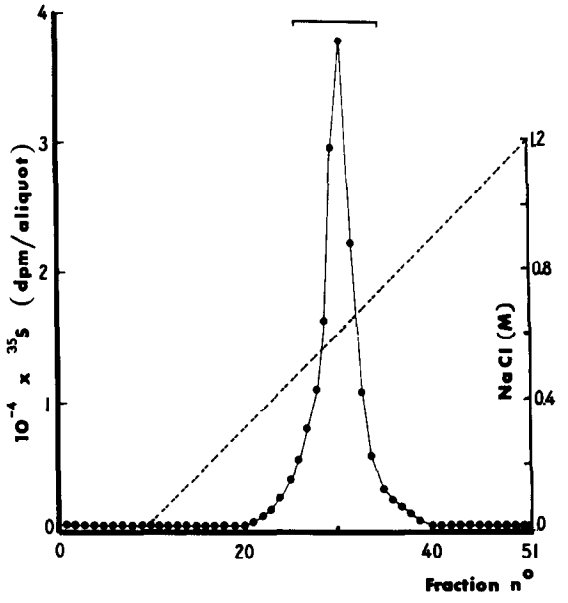

Fig.1. DEAE 52-cellulose chromatography of urea-soluble KC extracts after incubation with radiolabelled sulphate. 4 M guanidinium chloride-soluble cellular extracts corresponding to 150 × 10^6 KCs were dialysed against 7 M urea. The urea-soluble fraction was applied to a DEAE-cellulose column (1.1 × 5 cm) previously equilibrated with 7 M urea in 0.05 M Tris-HCl buffer (pH 6.8). Elution was performed with a 0–1.2 M NaCl linear gradient (----). The flow rate was 25 ml/h and 1 ml fractions were collected. The elution profile (——) was obtained with 0.1 ml aliquots. [^{35}S]Sulphated fractions (26–34) (thin bar) were pooled, dialysed and freeze-dried for further studies.

3.3. *Gel filtration of the radiosulphated DEAE-cellulose peak on Sepharose CL 6B*

After Sepharose CL 6B filtration under dissociative conditions (fig.3), a single peak was eluted near the void volume (K_{av} = 0.05).

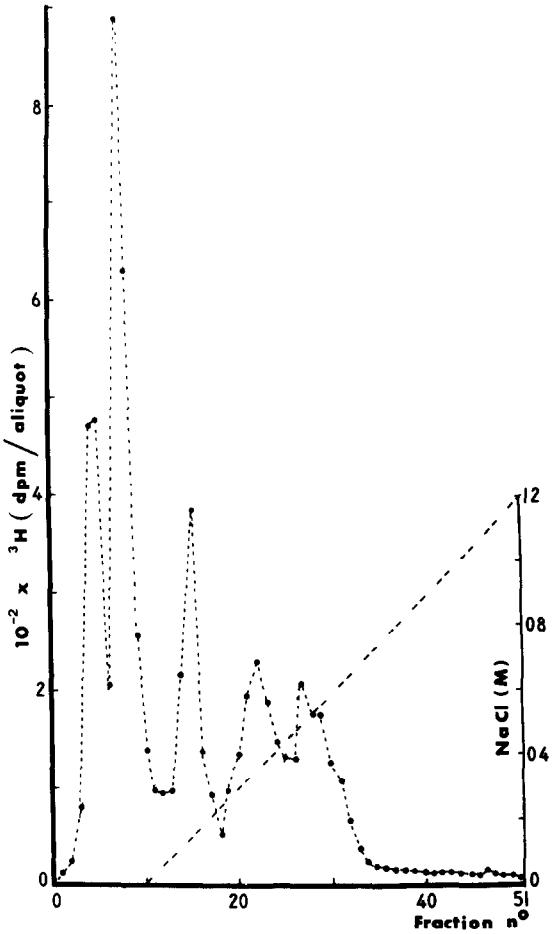

Fig.2. DEAE 52-cellulose chromatography of urea-soluble KC extracts after incubation with radiolabelled glucosamine. KC were incubated with D-[1-^{3}H]glucosamine. Chromatography was performed as described in fig.1. The last peak, eluted with 0.55 M NaCl, corresponds with the sulphated peak of fig.1.

3.4. *Effect of chondroitinases on the hydrodynamic size of the radiosulphated PG*

Aliquots of ^{35}S-labelled glycoconjugates eluting first at 0.55 M NaCl on DEAE-cellulose and then at K_{av} = 0.05 on Sepharose CL 6B, were submitted to chondroitinase ABC or AC digestion and further gel filtration on the same Sepharose CL 6B column. The peak completely disappeared with both chondroitinase AC (fig.3) and chondroitinase ABC (not shown), and this was correlated with the appearance, near the total volume (K_{av} = 0.96), of a single peak of lower molecular mass, sulphated degradation products. Almost all the radioactivity contained in the PG was recovered in the oligosaccharides.

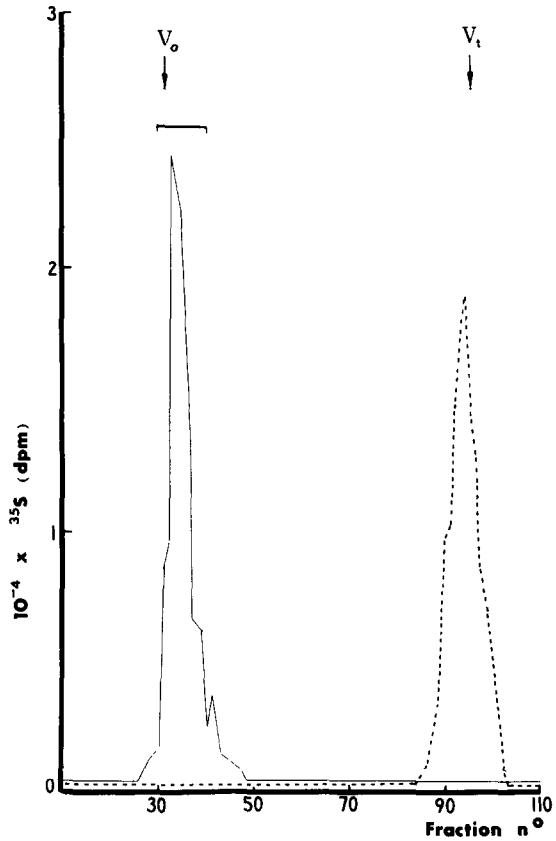

Fig.3. Effect of chondroitinase AC on the Sepharose CL 6B elution profile of the radiosulphated DEAE-cellulose peak illustrated in fig.1. The freeze-dried, sulphated sample was dissolved in 0.5 ml extraction buffer containing 4 M guanidinium chloride and applied to a column (0.7 × 42 cm) previously equilibrated with the same dissociative buffer. Material was eluted at a flow rate of 3 ml/h and 0.2 ml fractions were collected. The elution profile, indicated by (——), was obtained with 0.1 ml aliquots. After counting, the remaining material of the single peak at K_{av} = 0.05 was pooled (thin bar) and submitted to chondroitinase AC digestion for 18 h at 37°C. The elution profile indicated by (···) was obtained by rechromatography of the digested material under the same conditions. Radioactivity was determined on the whole fractions. The K_{av} = 0.05 peak was completely digested and the degradation products (oligosaccharides) appeared as a K_{av} = 0.96 peak. The arrows indicate the void volume (V_0) and the total volume (V_t) of the column.

3.5. *Electrophoretic pattern of the radiosulphated PG before and after chondroitinase digestion*

Fluorograms showed that almost all the CS-PG obtained from DEAE-cellulose chromatography was at the top of the stacking gels, and another band was observed at the top of the resolving gel (fig.4a,c). A similar blue pattern (not shown) was obtained with 'stains all' dye (BioRad) which specifically stains sulphated PG blue [24]. After chondroitinase AC (or ABC) treatment, there was a complete disappearance of the characteristic fluorographic pattern (fig.4b) and stains all blue staining pattern (not shown) of the sulphated Kurloff PG.

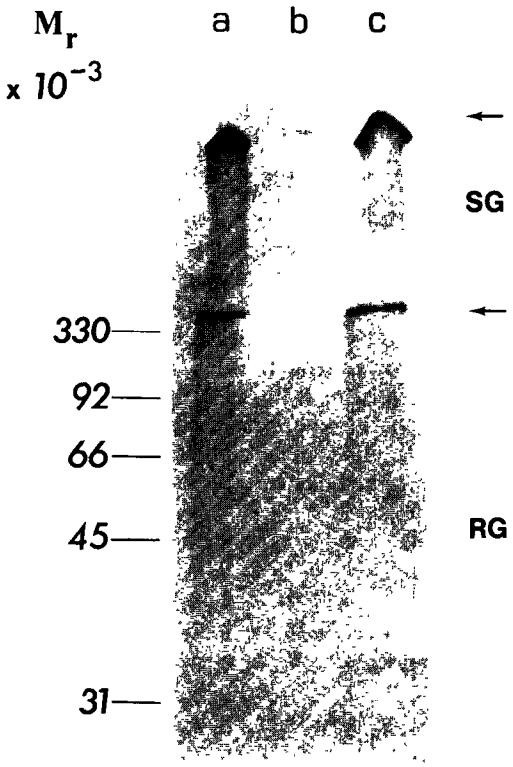

Fig.4. Effect of chondroitinase AC on the fluorographic pattern of purified Kurloff chondroitin [^{35}S]sulphate proteoglycan. Lanes a and c correspond to the sulphated peak from DEAE-cellulose chromatography. Lane b, corresponding to the same sample after digestion, shows the complete absence of labelling. After PAGE-10% SDS, the gels were silver stained, dried and exposed on Kodak X-Omat AR films for 30 days at −70°C. 3200 dpm were deposited per lane. M_r values indicated on the left were evaluated by the use of marker proteins. SG, stacking gel; RG, resolving gel.

4. DISCUSSION

The de novo synthesis of the CS-PG present in the KB by the KC themselves was therefore established for the first time. This finding reinforces the hypothesis of the in situ formation of the KB and excludes an endocytic origin. The results presented here indicate that the only sulphated PG synthesized by KC is chondroitin sulphate. Further analysis is being carried out in our laboratory to characterize this more precisely, though published data [5,6] lead us to conclude that the sulphated KC-PG is probably chondroitin-4-sulphate. This inventory of KC-PG should not be regarded as complete. Non-sulphated PG have not yet been examined. The elution of the sulphated Kurloff PG with 0.55 M NaCl corresponds to a highly anionic PG such as CS-PG. Nevertheless our best criterion for its identification is the high specificity of chondroitinase AC [25]. The $K_{av} = 0.05$ observed for the sulphated Kurloff PG is in agreement with its low penetration in acrylamide gels. The significance of the presence of CS-PG in other leukocytes and, a fortiori, in the enigmatic KC is uncertain. As intracellular (and particularly intralysosomal) CS-PG are involved in natural killer cytotoxicity [26], the natural killer activity of KC [27,28] could be related to the Kurloff CS-PG. The simultaneous presence in KB of acid phosphatases [3,29] and other acid hydrolases (Buat et al., unpublished) as observed in numerous other leukocytic granules [30–32] suggests that CS-PG are involved in enzymatic storage [33]. Such findings would confirm the hypothesis of a lysosomal nature of the KB [3].

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