Glycoconjugate Journal 17, 795–805, 2000

Biosynthesis of glycosaminoglycans and proteoglycans by the lymph node

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Previous studies of hyaluronan uptake and catabolism by lymph nodes indicated that the nodes might also add some HA of low molecular weight to the unabsorbed fraction that passes through from afferent to efferent lymph vessels.

The ability of lymph nodes to synthesise HA and proteoglycans was therefore examined (i) by perfusion of [^{3}H] acetate through an afferent lymph vessel *in vivo*, and recovery of labeled products from the efferent lymph vessel and from the node after perfusion; and (ii) by tissue culture of lymph nodes with [^{3}H] acetate.

Perfusion of lymph nodes with [^{3}H] acetate *in situ* yielded: (a), in outflowing lymph, small amounts of chondroitin/dermatan sulfate within the first hour which continued to be produced for up to 24 h; heparin in the second hour and HA in the third. In the nodes removed 17 to 19 h later, equal amounts of hyaluronan and chondroitin/dermatan sulfate and heparan sulfate proteoglycans were detected. In the tissue culture of lymph nodes: (1) HA, heparin and proteoglycans of heparan sulfate and chondroitin/dermatan sulfate were released into the medium but in the cell extract only heparan sulfate proteoglycan was detected; and (ii) molecular weight of the released hyaluronan ranged widely but was mostly less than 4–5 $\times 10^5$ D; heparan sulfate proteoglycan was 2.8 $\times 10^4$ to 9.4 $\times 10^5$ D; heparin 7.9 $\times 10^4$ D and chondroitin sulfate 1.3 $\times 10^4$ D, suggesting that the chondrotin sulfate were released from their proteoglycans core by enzymic degradation.

It is concluded that lymph nodes can release HA, heparin, heparan sulfate and chondroitin/dermatan sulfate proteoglycans into efferent lymph but the amount of hyaluronan is likely to be small without immune or other stimulation and its molecular weight is lower than in other tissues.

Keywords: **chondroitin sulphate, glycosaminoglycans, heparin, hyaluronan, lymph node, proteoglycans, synthesis**

Introduction

Although extracellular matrix makes a relatively small contribution to the structure of the lymphatic system, the lymphatic circulation plays a major role in the turnover of extracellular matrix. For example, it carries significant amounts of hyaluronan (HA) from the tissues to the blood-stream [1] where HA is rapidly removed, mainly by catabolism in the liver [2]. It is, moreover, not merely a conduit for matrix components but lymph nodes are direct participants in the catabolic turnover of HA, and probably of other glycosaminoglycans [GAGs] [3]. Thus, infusion of isotopically labeled HA into the afferent vessels of lymph nodes showed that lymph nodes have a high capacity for its absorption and for its metabolic degradation [4]. Labeled HA polymers recovered from outflowing lymph were much smaller than those infused, indicating that the larger polymers were either preferentially absorbed, consistent with the behaviour of the HA receptor responsible for its uptake from the blood stream in the liver [5] or were subject to extracellular degradation in passage through the nodes; or both. All these effects were reduced by concurrent infusion of proteoglycan (PG) fractions and their chondroitin sulphate (CS) chains [6], which suggests that both glycosaminoglycans share the same pathway for uptake and degradation in the lymph node, as they do in the liver.

These findings have been supported by analyses of lymph before and after passage through lymph nodes, which has revealed a reduction in GAG content mostly between 40% and 95%, and a reduction of similar or greater degree in both the weight-average and number-average (Mw) of HA [3]. In these studies, however, two pairs of samples showed lower PG content but little or no lowering in HA content of postnodal lymph although its Mw was lower as in other pairs. This might have been explained by the afferent and efferent samples of

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lymph having arisen from different tissues, such as skin, muscle or joints, despite their origin in the same field of lymph node drainage.

A local increase in the content of HA characterises the interstitial fluid in delayed hypersensitivity reactions induced in the lung [7] or dermis [8] and in the latter case, HA is also increased in lymph [9]. It therefore seemed possible that the normal level of immunological activity in the lymph node similarly promotes synthesis of HA, which could readily enter outflowing lymph through the loosely structured channels in the node.

This question has been examined by analysis of outflowing lymph during perfusion of lymph nodes *in vivo* with [^{3}H] acetate, introduced through an afferent lymph vessel close to the node. In the light of the results, the ability of lymph nodes to synthesise HA and proteoglycans was further investigated by tissue culture.

Materials and methods

GAG and PG biosynthesis *in vivo*

Surgical preparation of lymph nodes for perfusion

This was done in Merino ewes as described for perfusion with labeled HA [4]. The efferent and one of the several afferent lymph vessels to a popliteal node were cannulated for the purpose.

[^{3}H] acetate perfusion of lymph node

Twenty-four hours after surgery the intubated afferent was perfused with Dulbecco PBS containing BSA (10 mg/ml, Sigma, St Louis, USA), 9 mM glucose and [^{3}H] acetate (400 μCi/ml Amersham, Bucks, UK). The efferent lymph was drained at hourly intervals into sterile tubes containing EDTA, while overnight lymph collections contained 0.02% sodium azide and EDTA. On completion of each study, the perfused popliteal node was removed within 5 min of death, weighed and stored at −70°C until analysed. The deadspace volume of the infusion line was measured, and the mean perfusion rate calculated from the measured loss from the perfusate reservoir.

Collection and analysis of lymph

The lymph was centrifuged in a Beckman TJ-6 centrifuge at 400 g_{av} for 10 min, and the cell free lymph retained. Lymph pellets were washed by centrifugation in 2 ml of PBS, resuspended in 0.1 ml of water and dissolved in 1 ml NCS tissue solubiliser (Amersham, Bucks, UK) to which 10 ml of scintillant was added. The pellets contained background quantities of radioactivity. The cell-free lymph was dialysed (Mw exclusion 6000–8000 D) against 5 changes of 500 volumes of 10 mM Tris/saline pH 7.4, at 4°C, to remove unincorporated [^{3}H] acetate. [^{3}H] activity in the dialysed lymph was determined in triplicate 100 μl samples, after dilution to 1 ml with distilled water and addition of 10 ml of scintillant. The lymph was stored at 4°C with 0.02% sodium azide until GAG and PG analysis.

Collection and analysis of blood

In Expts 1–3 where lymph nodes were perfused with [^{3}H] acetate, blood samples were taken hourly for 1–5 h or 1–7 h and at 24 h, in order to detect possible diffusion of [^{3}H] acetate from the infused lymph channels to the bloodstream and subsequently tissue fluids. The blood was collected into sterile EDTA tubes, and centrifuged in a Beckman TJ-6 centrifuge at 400 g_{av} for 10 min. Triplicate 100 μl samples of plasma were diluted to 1 ml with distilled water before the addition of 10 ml of scintillant.

GAG and PG biosynthesis *in vitro*

Preparation of lymph node tissue cultures

Each node was vertically halved and weighed, and each half used for 5 h and 20 h cultures respectively. Each half was cut into pieces of approximately 2 mm^3. The suspension was transferred into a 50 ml tube, in which the deposit was twice washed by resuspension in RPMI 1640 (Sigma, St Louis, USA) at 37°C after centrifugation in a Beckman TJ-6 centrifuge at 500 g_{av} for 10 min. The supernatant containing free lipid was aspirated from the pellet. The pellet was resuspended in culture medium and continuously agitated to ensure accurate and even distribution of tissue during division into separate cultures of 10 ml volume. The culture medium consisted of RPMI 1640, with 20% FCS, 9 mM glucose, 20 mM sodium bicarbonate, 40 μg/ml gentamicin and 60 μCi/ml [^{3}H] acetic acid. The cultures contained greater than 10^8 cells/10 ml in Petri dishes of 100 mm diameter and 15 mm depth. Cultures were incubated in the medium at 37°C in 5% CO_2 for 5 and 20 h. At these times, cell and tissue pieces were separated from the conditioned media by centrifugation in a Beckman TJ-6 centrifuge at 500 g_{av} for 15 min. The conditioned medium and cell layer was subjected to further purification and GAG analysis.

Interaction between the [^{3}H] acetate infusate and lymph proteins

The perfusate and afferent lymph were chromatographed on Sephadex G-50 before and after dialysis (6000–8000 Mw exclusion) against 10 mM Tris/saline/0.02% sodium azide pH 7.4 at 4°C.

Glycosaminoglycan and proteoglycan analysis (see Figure 1 for summary of methodology)

After each of the degradative treatments described below, samples of 1 ml each were subjected to size exclusion chromatography in Sephadex G-50 columns (1.6 cm × 30 cm) 30 cm) equilibrated in 0.15 M NaCl/phosphate pH 7.25 that contained 19 mM NaH_2PO_4, 38 mM Na_2HPO_4 and 94 mM NaCl and eluted at 13.6 ml/h in fractions of 2 ml. After

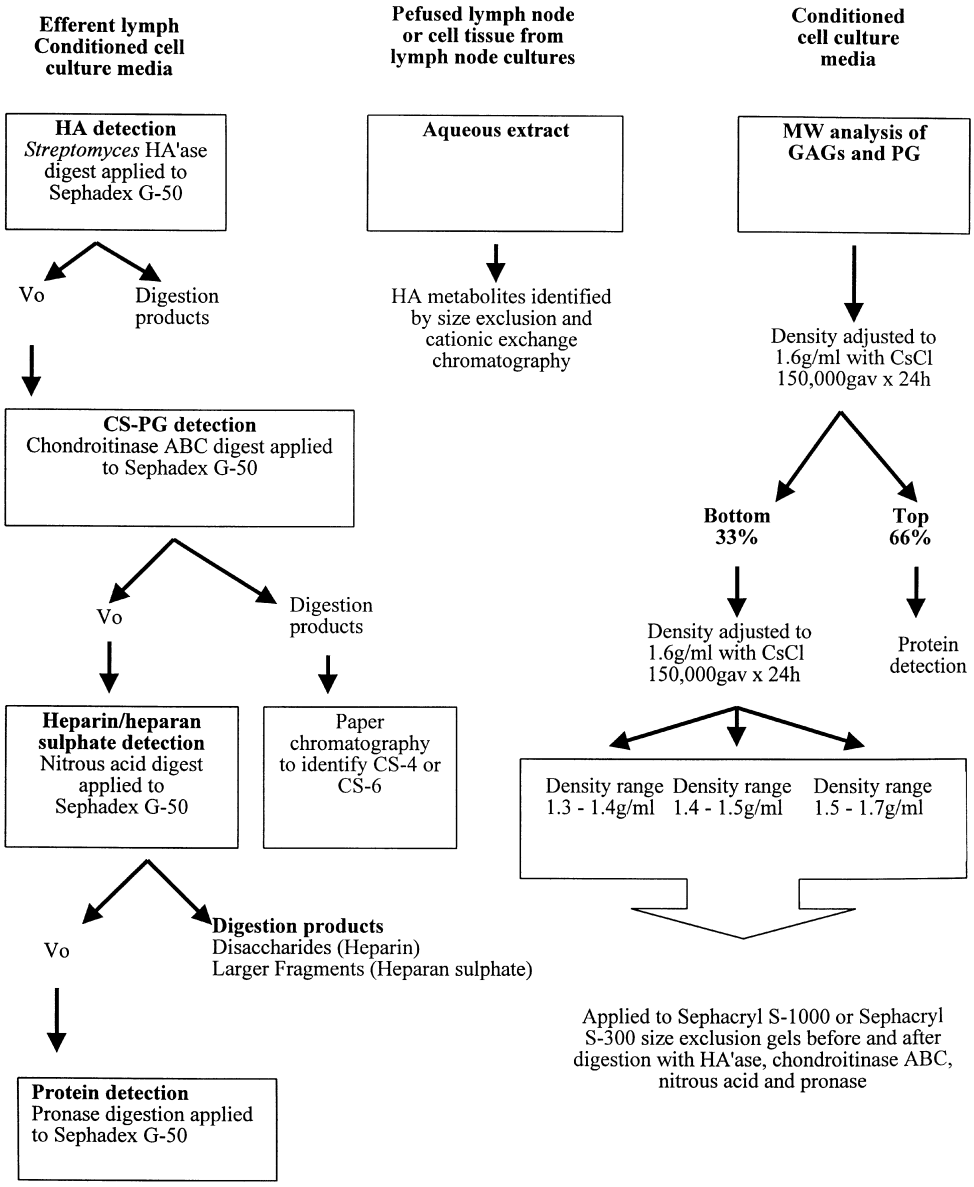

Figure 1. Diagrammatic representation of methodology for GAG and PG analysis.

determination of radioactive content in 0.5 ml volumes of each fraction, the residue at V_o was recovered for the next step in identification. The degree of degradation in each treatment was calculated as a fraction of the total eluted ^3H.

Hyaluronan: The presence of [^{3}H]HA in an unknown sample was determined by digestion with *Streptomyces hyalurolyticus* hyaluronidase (Calbiochem, Germany). The enzyme was reconstituted in 9 mM KH_2PO_4 and 7 mM $Na_2HPO_4.2H_2O$, pH 6, and 5 turbidity reducing agents (TRU) was added to the sample at 0 and 3 h. The digestion mixture was incubated at 37°C for 24 h. Non-radioactive HA was quantitated by a HA radio-metric assay (UpJohn Pharmacia, Sweden).

Chondroitin sulphate/dermatan sulphate proteoglycans (CS/DS-PG): Samples were incubated with 0.1 unit *Proteus vulgaris* chondroitinase ABC (Sigma, St Louis, USA) in 50 mM Tris/HCl, pH8, for 24 h at 37°C. The chondroitinase ABC was shown to be hyaluronidase-free. The digest was rechromatographed on Sephadex G-50 to estimate the [^{3}H] CS content of the sample. Non-radioactive sulphated proteoglycans were quantitated with the 1,9-dimethyl-methylene blue (DMB) dye-binding assay [10].

[^{3}H]Heparan sulphate proteoglycans (HS-PG) and heparin (HEP): A single treatment with nitrous acid selectively degrades the *N*-sulphated GAGs, heparins and heparan sulphate [11]. Heparin contains a relatively high proportion

of *L*-iduronic acid and *N*-sulphated glucosamine residues (80% c.f. 50% in HS), so that nitrous acid generates small fragments, predominantly disaccharides. Heparan sulfate on the other hand, contains short uninterrupted sequences of *N*-acetylated or *N*-sulphated disaccharides so that deaminative cleavage by nitrous acid generates fragments of various sizes [12]. To perform nitrous acid degradation, one ml of aqueous sample was mixed with 0.364 ml of 1 M $NaNO_2$ and 0.152 ml of glacial acetic acid. This mixture was allowed to react at 25°C for 12 h, at which time 0.515 ml of 1 M ammonium sulphamate was added [13].

Protein: Pronase (Sigma, St Louis, USA) was added to samples to a final concentration of 2 mg/ml in 0.1 M Tris-HCl pH 8, 0.5 mM $CaCl_2$, heated at 37°C for 24 h [14].

Calculation of [^{3}H]HA and [^{3}H]CS/DS-PG specific activity

The lymph node cultures were labeled for 5 and 20 h *in vitro* with 60 μCi/mL of [^{3}H] acetate. The cell medium was fractionated in an associative CsCl density gradient with a starting density of 1.6 g/mL. The bottom 33% of each tube was analysed for GAGs and PG as previously described, and the top 67% was digested with pronase and identified as protein. The incorporation of [^{3}H] into HA was determined by the non-dialysable (Mw exclusion 6000–8000 D) dpm which was susceptible to digestion by 10 TRU of *Streptomyces* hyaluronidase at pH 6, 37°C for 24 h. Digested material was separated from *Streptomyces* hyaluronidase resistant material by Sephadex G50 gel filtration. The percentage of HA was calculated as the proportion of material which was displaced to Kav's 0.66 and 0.77, while the μg quantity of HA produced by the node was determined using the HA radiometric assay after correction for the amount of HA derived from the culture medium. The specific activity of the [^{3}H]HA was calculated by dividing the total [^{3}H] dpm associated with [^{3}H]HA by the μg amount of HA. The CS/DS-PG specific activity was calculated by the same method where CS/DS-PG and was detected by digestion with *Proteus vulgaris* chondroitinase ABC and the μg quantity of CS/DS-PG determined by the DMB assay.

Characterisation of chondroitin sulphate disaccharides

Fifty μl containing [^{3}H]CS-PG disaccharides generated by chondroitinase ABC digestion were applied to Whatman 3 M filter paper with [^{3}H]disaccharide standards (kindly supplied by Dr C. Robinson, Monash University, Australia) applied to outside lanes of each paper chromatogram. Chromatograms were run for 24 h in a descending solvent system of isobutyric acid: 2 M NH_3 (5 : 3 v/v), air-dried, stained with silver [15] and fixed with 2 M NH_4OH. The ratio of [^{3}H] C4S to [^{3}H] C6S was determined by counting β-emission after a distilled water extraction of the chromatogram (1 × 2 cm sections).

Molecular weight analysis of [^{3}H] GAG and PG in conditioned media

Conditioned media were adjusted to density 1.60 g/ml by the addition of 0.816 g CsCl/ml of media and centrifuged in a Beckman L5-65 ultracentrifuge at 150 000 g_{av} for 20–24 h at 4°C (associative conditions). The bottom third (density > 1.73 g/ml) and top two-thirds (density 1.50–1.73 g/ml) of the tubes' contents were recovered by downward displacement with paraffin oil. The content of one tube from each batch was fractionated in 1 ml aliquots, whose density and radioactivity were measured to ensure that the fractions had the correct density for the distribution of PG, HA and other glycoproteins. The fractions were dialysed against 10 mM Tris-HCl/0.15 M sodium chloride/0.02% sodium azide pH 7.4 at 4°C. On completion of dialysis radioactivity in each fraction was measured by counting triplicate 100 μl volumes. In Expts. 5 to 7, the high-density fractions recovered in associative conditions were adjusted with CsCl and buffered GuHCl to a density of 1.45 g/ml and 4 M GuHCl, pH 7.4 (dissociative conditions). Gradients were generated again at 150 000 g_{av} for 20–24 h at 4°C. Density and ^{3}H activity were determined in 2 ml fractions. To ensure complete separation of HA (density range 1.44–1.50 g/ml) from CS-PG (density 1.52–1.66 g/ml), fractions of density 1.44–1.66 g/ml were pooled and another dissociative gradient was regenerated in the same conditions. Final fractions of 1 ml were pooled according to the above densities. Those of low density (< 1.41 g/ml) were examined to detect any residual labeled proteins from the initial associative separation.

HA: To determine whether any [^{3}H]-labeled macromolecules other than HA were present in the 1.44–1.50 g/ml density range, a *Streptomyces* hyaluronidase digestion was performed. An aliquot of any *Streptomyces* hyaluronidase-resistant material was subjected to sequential degradation by 0.1 U *Proteus vulgaris* chondroitinase ABC and nitrous acid which demonstrated that a heparan sulphate (as assumed by the nitrous acid generation of fragments of various molecular weights) of density range 1.44–1.50 g/ml was synthesised. Two ml of the purified media (density range 1.44–1.50 g/ml) were applied to a Sephacryl S-1000 eluted in 0.15 M NaCl/phosphate pH 7.25 which contained 19 mM NaH_2PO_4, 38 mM Na_2HPO_4 and 94 mM NaCl at 13.6 ml/h. In experiments where molecules of < 70 kD were present a more discriminating analysis was performed in Sephacryl S-300 and Sephadex G-50. All Mw estimations were calculated using calibration data for HA in Sephacryl S-1000 provided by Dr K Granath and in Sephacryl S-300 [2].

Proteoglycans: The fraction within the dissociative density range of 1.52–1.66 g/ml was pooled and analysed to determine the purity and Mw of CS/DS-PG. Samples containing 10 000–20 000 dpm/2 ml were applied to Sephacryl S-1000 which was eluted in 4 M GuHCl buffered with 50 mM Tris/HCl pH 7.4, flow-rate 13.6 ml/h. The column fractions

were monitored for radioactivity by counting 0.5 ml of sample to which 0.5 ml of absolute ethanol and 4 ml of aqueous scintillant was added. To demonstrate that the CsCl gradient had separated HA from CS/DS-PG and heparin/heparan sulphate proteoglycans (HEP/HS-PG) at density 1.52–1.66 g/ml, the gradient fractions of density 1.52–1.66 g/ml were sequentially digested with 0.1 U *Proteus vulgaris* chondroitinase ABC followed by nitrous acid degradation as previously described. The digested samples were chromatographed on Superose 12, with the percentage of PG being calculated as the percentage of PG material shifted from V_o to the region of disaccharides or intermediate oligosaccharides.

Detection of cell-associated [^{3}H] GAG and PG

Each perfused lymph node or the cell/tissue from *in vitro* experiments was dispersed at a 1 : 2 w/w ratio in chilled 0.15 M phosphate/NaCl buffer and homogenised twice for 1 min in a Polytron PCU-2, then held at 4°C for 72 h, and agitated every 24 h. After 3 cycles of freezing and thawing the extract was clarified by centrifugation in a Beckman L65-5 ultracentrifuge at 113 000 g_{av} for 2 h, 4°C. Centrifugation resulted in a variable volume of lipid at the top of the tube, which was aspirated from the supernatant. Any cellular residue was further extracted with Triton X-100 to ensure complete recovery of all cell-associated GAG and proteoglycan. This extraction produced negligible radioactivity, indicating that the aqueous extraction was highly efficient. The aqueous lymph node extract was dialysed and the GAG content determined as previously described.

Results

GAG and PG synthesis *in vivo*

Incorporation of [^{3}H] acetate in macromolecular content of lymph (Figure 2A)

The incorporation of [^{3}H] acetate in non-dialysable macromolecules was very low; the percentage recovery of [^{3}H] as macromolecules ranging from 0.08 to 0.84% (mean ± SD: 0.29 ± 0.30) of the total infused ^{3}H. The low incorporation was not due to unavailability through binding of [^{3}H] acetate to albumin in the perfusate or afferent lymph, as this accounted for 0.48% of the total [^{3}H] acetate in the perfusate. As seen in Figure 2A the major incorporation of [^{3}H] into synthetic product occurred from 0 to 5 h, at which time a steady state of radioactive precursor incorporation was observed for the 7–24 h collection periods.

Recovery of [^{3}H] from blood during perfusion of lymph nodes

The diffusibility of [^{3}H] acetate as a synthetic precursor is clearly a problem in metabolic studies *in vivo*. To gain some idea of the loss of infused [^{3}H] acetate by diffusion into fluids, serial plasma samples were studied in Expts. 1–3 of the [^{3}H] acetate perfused lymph nodes. There was a linear increase in plasma radioactivity during the infusion period, with only a slight drop in plasma [^{3}H] after infusion ceased (data not shown). [^{3}H] appeared initially in plasma as both acetate and water. The 1 h plasma contained [^{3}H] acetate and ^3H_2O, whereas the 5 h plasma contained ^3H_2O, but little or no [^{3}H] acetate (data not shown). These results were substantiated by chromatography in Bio-Rad HPX-87H cation exchange resin. Chromatography of the perfusate in Sephadex G-50 confirmed absence of ^3H_2O. It is therefore valid to assume that the appearance of labeled water in the plasma was clearly due to metabolic oxidation of the acetate. Since [^{3}H] acetate is rapidly converted to ^3H_2O after intravenous injection, the slight fall in plasma activity at 24 h in Expt. 3 indicates that the dispersal of ^{3}H from plasma to total body water was already quite extensive during the infusion of the node.

Identity of [^{3}H]-labeled macromolecular material from lymph (Figure 2B)

The majority of [^{3}H] acetate was incorporated into protein (range: 48–91%, mean ± SD: 74% ± 15%). The remainder of the [^{3}H] acetate was used as a synthetic percursor of:

[^{3}H]HA: The labeled HA content of efferent lymph is illustrated in Figure 2B where the individually synthesises macromolecules are expressed as a percentage of the total dpm of the labeled synthetic products, enabling quantitative comparisons to be made between experiments. Small amounts of HA were detected in 6 of 29 samples. No HA was found after the infusion was ceased.

[^{3}H]CS/DS-PG: Due to the sequential nature of the analysis, there was often insufficient sample for CS/DS-PG detection. There was not enough incorporation of [^{3}H] into CS-PG released from the node *in vivo* to enable characterisation of the CS/DS-PG disaccharides.

[^{3}H]heparin/heparan sulphate proteoglycan: The nitrous acid digestion products of the efferent lymph were uniformly in the range of disaccharides, indicating them to be heparin.

[^{3}H]-labeled residues in node on cessation of infusion: The amount of cell/tissue associated [^{3}H] varied between experiments, ranging from 0.11 to 7.97×10^5 dpm/g nodal tissue. The major difference in cell/tissue associated GAGs and the GAGs released into efferent lymph was in the nitrous acid-susceptible fractions, which showed that the cell/tissue associated sulfated GAG from the lymph node included HS-PG, but only heparin was released into the efferent lymph.

Sephadex G-50 and Aminex HPX-87H HPLC cation exchange analysis of undialysed aqueous node extract identified ^{3}H-labeled GlcNAc, acetate and water. The recovery of labeled GlcNAc suggests that there may be concurrent degradation of endogenous GAG, but is incomplete evidence in the absence of labeled GlcNAc-6P. Figure 2B demonstrates the percentage composition of GAGs synthesises by the node while utilizing [^{3}H] acetate as a precursor, but due to the

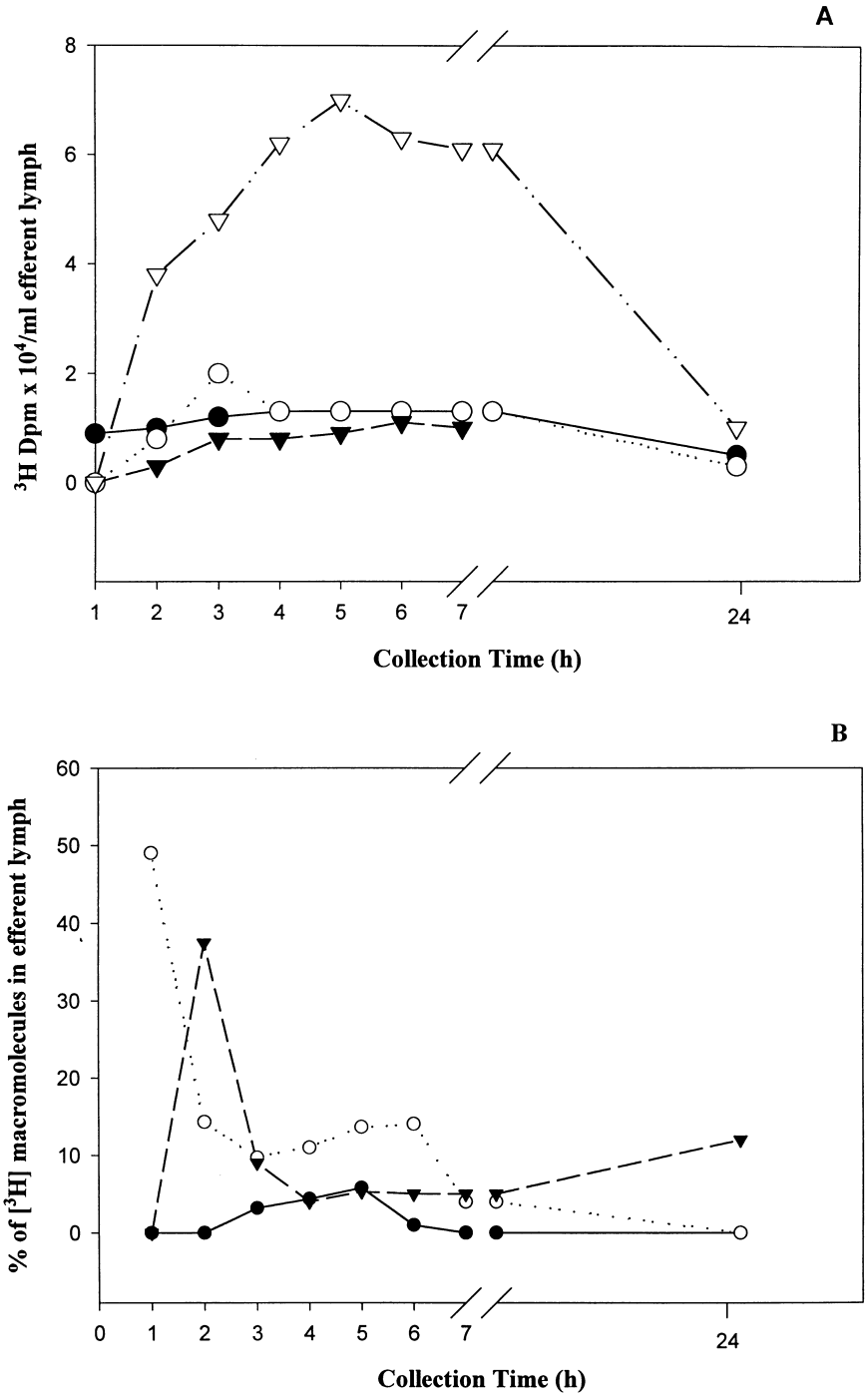

Figure 2. A ^{3}H recovered in non-dialysable content of efferent lymph from perfused lymph node. The cannulated afferent vessel was perfused with 400 μCi/mL [^{3}H] acetate for 1–5 or 6 h. Lymph was collected hourly for 5–7 h in experiments 1–4 (as described in Materials and methods), and followed by an overnight collection in experiments 2–4. The collected efferent lymph was exhaustively dialysed before measurement of its radioactivity as previously described. The data represents the non-dialysable radioactivity at each time point, where (▼) Expt. 1, (●) Expt. 2, (○) Expt. 3 and (▽) Expt. 4. **B** Release of [^{3}H] HA, [^{3}H] CS/DS-PG and [^{3}H] heparin into efferent lymph during and after perfusion of lymph nodes with [^{3}H] acetate The efferent [^{3}H] acetate perfusate was collected at hourly intervals, and cells were removed by centrifugation in a Beckman TJ-6 centrifuge at 400 g_{av} for 10 min. The cell-free lymph was exhaustively dialysed to remove unincorporated [^{3}H] acetate. The lymph was sequentially digested with *Streptomyces* hyaluronidase and *Proteus vulgaris* chondroitinase ABC. After gel filtration chromatography on Sephadex G-50 any material remaining at V_o was pooled and subjected to nitrous acid degradation. The dpm of [^{3}H] individual GAG and PG in the lymph were calculated as the proportion which was displaced from V_o in Sephadex G-50. The [^{3}H] GAG and PG are expressed as a percentage of the non-dialysable [^{3}H] dpm present in the lymph, where each point is the mean value of five separate determinations. (●) HA, (○) CS/DS-PG and (▽) HCP/HS-PG.

possibility of continuous metabolism in the node, these are only representative of the composition when the node was excised.

GAG and PG synthesis *in vitro*

GAG and PG recovery from the lymph node conditioned medium

[^{3}H]HA: Table 1 summarises the incorporation of [^{3}H] acetate into [^{3}H]HA released into the media. Although the total amount and specific activity of HA differed between 5 and 20 h it was evident that the incorporation of [^{3}H] acetate into the N-acetyl glucosamine moiety of HA increased significantly after a further 15 h of incubation.

[^{3}H]CS/DS-PG: Analysis of the CS/DS disaccharides revealed that the 5 h media contained a higher percentage of C4S, while the 20 h contained a greater proportion of C6S. In the cell-associated fractions, the C6S was two-fold greater than the C4 sulphate at both 5 h and 20 h and in the perfused lymph node on cessation of perfusion.

[^{3}H]Heparin/heparan sulphate proteoglycan: The conditioned media from the lymph node cultures contained both HEP and HS-PG, while HS was identified as the only cellassociated *N*-sulphated GAG.

Molecular weight analysis of GAGs and PG synthesised and released by the lymph node in vitro (Table 1)

Molecular weight analysis of [^{3}H] GAGs and PG synthesised by the lymph node could only be performed on the macromolecules produced by the node *in vitro*, due to insufficient radioactivity and quantity of labeled GAGs and PG in efferent lymph.

[^{3}H]HA: (Figure 3) After cesium chloride centrifugation under dissociative conditions it was possible to calculate the proportion of dpm present in the central density band of the gradient which was susceptible to *Streptomyces* hyaluronidase and nitrous acid. The labeled material in the central density band was subjected to chromatography in Sephacryl S-1000 (HA fractionation range $\simeq 7 \times 10^4$ D to $> 7 \times 10^6$ D) and in Sephacryl S-300 which discriminates HA in the range 2×10^3 to 6.7×10^4 D. Molecular weight was determined by comparing the percentage of *Streptomyces* hyaluronidase-susceptible material and the shift in Mw profile before and after enzyme digestion. Molecular weight analysis of both the macromolecules produced at both 5 and 20 h, demonstrated that the lymph node synthesises HA of various Mw, ranging from 7×10^6 to 3×10^4 D. A nitrous acid-susceptible molecular species of 2.8×10^4 D was observed in the media removed at both 5 and 20 h of incubation. Nitrous acid digestion products were polydisperse, indicating HS-PG. The polydispersity of the HA synthesises by the lymph node cultures suggested post-synthetic degradation, which was confirmed by further analysis in Sephadex G-50. The G-50 analysis (data not shown) brought to light the presence of di- and tetrasaccharides. The [^{3}H]HA synthesised by the lymph node is possibly degraded by a combination of free radical and enzymic activity, or merely the action of a tissue hyaluronidase which hydrolyses the endo-N-acetyl hexosamine bonds of HA.

Other [^{3}H]PG: The combination of Sephacryl S-1000 analysis and the percentage of material susceptible to enzyme or nitrous acid suggested that the molecular weight of HEP/HS-PG was approximately 2.8×10^4 D and 6.6–9.4×10^5 D (as equivalent to HA calibration data) while the CS/DS-PG was presumably degraded by proteases or cellular degradation during the purification procedure resulting in species of 1.3×10^4 D.

Table 1. Quantitation and characterisation of [^{3}H] GAGs and proteoglycans synthesised by the lymph node *in vitro*

	Hyaluronan		*Chondroitin/Dermatan sulfate proteoglycans*		*Heparin/heparan sulfate proteoglycans*		*Protein*	
	5 h	*20 h*	*5 h*	*20 h*	*5 h*	*20 h*	*5 h*	*20 h*
Conditioned media (% of total [^{3}H] molecules)	17.4 ± 14.8	43.1 ± 17.3	58.6 ± 13.1	30.3 ± 13.1	3.3 ± 5.2	16.7 ± 16.5	21.1 ± 12.6	19.4 ± 9.7
Cell/tissue (% of total [^{3}H] molecules)	0	1 ± 1.5	14.0 ± 15.0	12.6 ± 15.8	15.4 ± 20.5 heparan sulfate	10.2 ± 4.1 heparan sulfate	78.2 ± 20.2	77.2 ± 19.6
Modal Mw of [^{3}H] molecules in conditioned media	7×10^4 to 1.5×10^6	7×10^4 to 7×10^6	1.3×10^4	1.3×10^4	2.8×10^4 to 6.6×10^5	2.8×10^4 to 9.4×10^5	NA	NA
Specific activity of [^{3}H] molecules in conditioned media	14 446 ± 12 666	81 699 ± 98 724	11 024 ± 11 416	5056 ± 4781	NA	NA	NA	NA

NA is an abbreviation for Not Analysed.

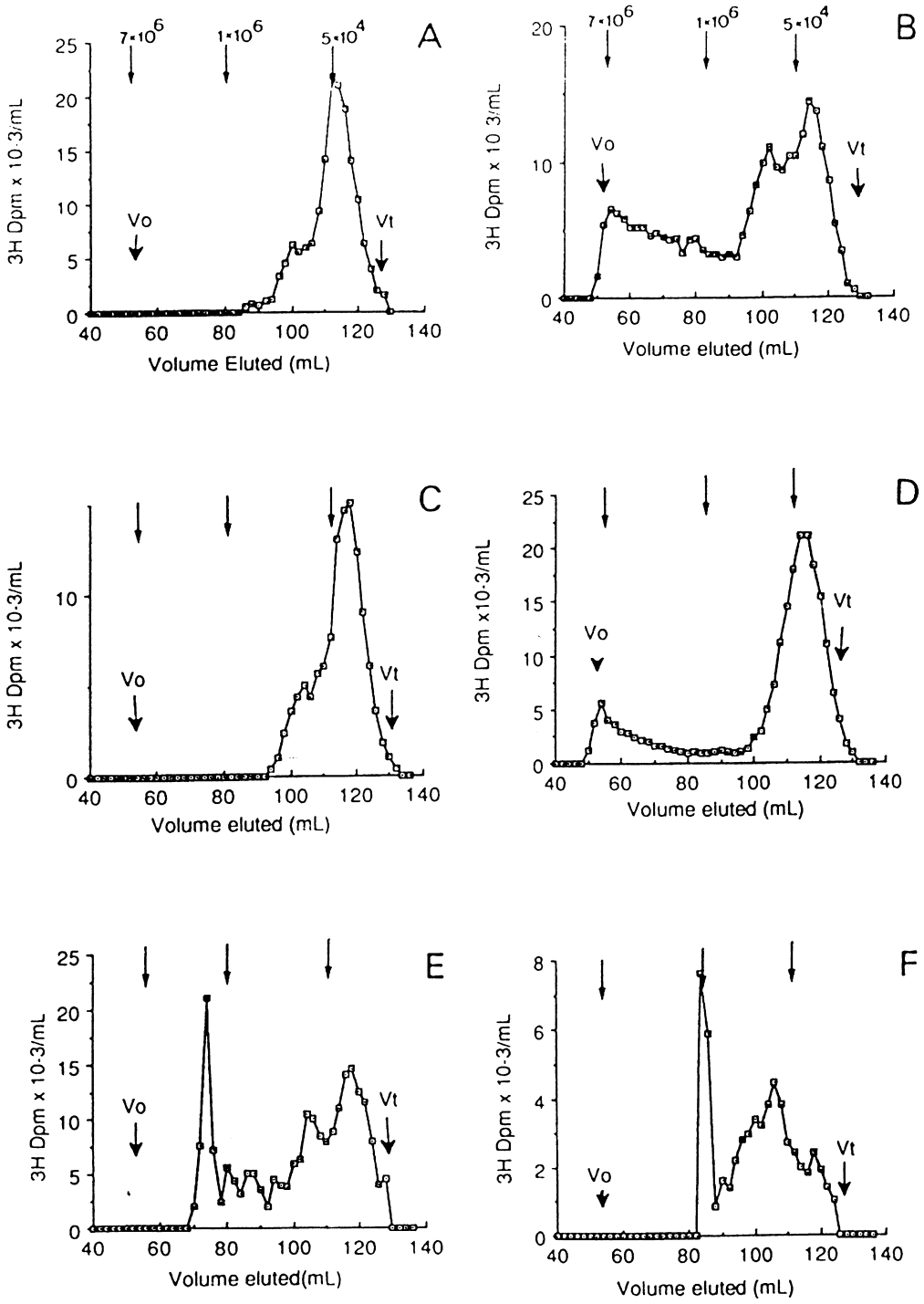

Figure 3 Molecular weight analysis of hyaluronan synthesised by cultured lymph node tissue. Conditioned media from lymph node cultures were subjected to 2 dissociative CsCl gradients. The density range of 1.44–1.50 was pooled and subjected to sequential enzymic digestions to identify macromolecular products. On identification of any [^{3}H] species the purified medium was applied to a Sephacryl S1000 gel. The elution profiles were compared to calibration data to determine relative Mw of [^{3}H] molecules. The Mw of the HA synthesises by lymph nodes *in vitro* after a 5 h incubation (A, C and E) are compared to a 20 h incubation (B, D and F).

Cell-associated [^{3}H]glycosaminoglycans and proteoglycans produced by the lymph node cultures

The incorporation of [^{3}H] acetate into cell-associated GAGs and PG by the node *in vitro* is summarised in Table 1. HA was synthesised by 5 h cultures but it was rapidly and completely liberated into the media since no cell associated HA was found in the cell pellet. At 20 h only 0–4% of the total [^{3}H] activity was cell-associated. At 5 and 20 h the cellular fraction of the

lymph node contained a very small proportion (3–19%) of cell-associated radioactivity of CS/DS-PG, with the exception found in Expt. 6, where 44% of [^{3}H] activity extracted at 20 h was identified as CS/DS-PG. The digestion products generated after *Proteus vulgaris* chondroitinase ABC treatment were identified as both C4S and C6S with the greater proportion (60–85%) being C6S. The percentage of [^{3}H] dpm identified as HEP/HS-PG was in the range of 0–56% where the nitrous acid cleavage products were shown to polydisperse so suggesting the macromolecule was HS-PG instead of heparin.

Discussion

Through sequential enzymic digestions it has been possible to demonstrate both *in vivo* and *in vitro* that the lymph node produces GAGs and PG. The incorporation of [^{3}H] acetate by the perfused node into efferent lymph or cell-associated GAGs and PG was very low with only 0.08%–0.84% (avg. 0.3%) of the [^{3}H] precursor incorporated in GAG of efferent lymph and 0.005%–0.07% (avg. 0.02%) in tissue/cell-associated macromolecules. The degree of non-specific absorption of the [^{3}H] acetate to proteins in lymph or perfusate was shown to be negligible (0.48% of total perfusate activity), substantiating the availability of [^{3}H] acetate as a synthetic precursor. As a consequence of its low Mw and solubility in both aqueous and lipid phases of tissue and the high normal blood flow through lymph nodes, infused acetate would be removed from the node at a high rate by diffusion into blood capillaries. The variations in the rates of output of synthetic product during the infusion might therefore depend on blood flow in the particular node, and on the concentration of [^{3}H] acetate established by diffusion to and away from the site of cellular synthesis. Since [^{3}H] acetate is not stored, and would rapidly disappear on cessation of infusion (by diffusion from the node and metabolism elsewhere in the body), the labeled macromolecular product recovered after 7 h would represent material synthesised during the infusion. Fluctuations in the rate of lymph flow (data not shown) did not appear to correlate with the rate of release of macromolecules.

The lymphatic system consists of a well defined network of vessels that collect and convey tissue fluid through the lymph node back to the bloodstream, acting as a fine adjustment in the circulation of water and plasma proteins between blood and tissue fluids. Tissue fluids are returned in lymph to the bloodstream at a rate of 3–4 L/d in a resting adult human, compared with a plasma circulation of approximately 5000 L/d. This estimate indicates the net fluid exchange from tissues, an exchange which would distribute the [^{3}H] acetate throughout body fluid and tissues, and subsequently reduce its availability as a synthetic source. The passive or facilitated fluid transport to the bloodstream was substantiated by the persistent [^{3}H] level in the plasma, where much of it was ^3H_2O.

The lymph nodes appeared to synthesise the PG and GAGs in a particular sequence; both in culture and *in situ* labeled CS/DS-PG appeared first, followed by HA. Of [^{3}H] incorporated in synthetic products in the 5 h node cultures an average 17.4% was found in HA, 58.6% in CS-PG and 8.0% in HEP/HS-PG. In the 20 h cultures, the corresponding figures were 43.1% in HA, 30.3% in CS/DS-PG and 21.8% in HEP/HS-PG.

It has been well established that monocytes [16], T lymphocytes, nature killer cells [17] and B lymphocytes [18] produce CS-PG. CS-PG appears to play a vital role in cell-mediated cytotoxicity where it protects natural killer cells from autolysis [19]. In the lymph node B lymphocytes are located primarily in the lymphoid follicles of the cortex, and T lymphocytes mainly in the paracortical area. The areas of lymphocyte aggregation are highly vascularized with lymphatic sinuses that would enable an exchange between lymph-borne synthetic precursors and lymphocytes, resulting in products such as CS-PG. The lining cells of the sinuses resemble the attenuated cells of blood vascular endothelium, which are closely associated with bands of stroma containing cells with fibroblastic characteristics [3]. Fibroblastic cells are commonly active producers of HA, which could also account for the identification of GAGs in efferent lymph and conditioned media.

There was a definite trend in the sulphation pattern of CS-PG. The 5 h media of the lymph node cultures contained predominantly C4S (53–84%), but after 20 h of incubation the cultures were shedding more C6S (64–71%). In the cell-associated CS-PG from nodes *in vivo* and *in vitro* there was always a predominance of C6S (60–85%), indicating a preferential initial release of C4S into the surrounding environment. The relative amounts of C4S and C6S could be determined by many factors. Ionic strength, pH, 3′-phosphoadenosine 5′-phosphosulphate (PAPS) concentration, incubation time, composition of buffer, as well as the sulphate acceptor, can affect the activity and type of sulphation [20]. For example, incubation of chick embryo cartilage at pH 6.5 with PAPS yielded a radioactive labelling of C6S (60–70%) and C4S (30–40%), while in incubations at pH 7.8, C6S is exclusively formed [20]. The lineage of cells producing CS could also account for differences in sulphation patterns, for leucocytes have been shown to produce C4S almost exclusively [21]. This would account for a slower release of C6S into the culture media or lymph despite the majority of cell-associated CS-PG being C6S. Considering that the lymph node capsule is predominantly collagen the CS/DS-PG which was identified in the efferent lymph, conditioned medium and cell-associated could have been the CS/DS-PG, decorin. Decorin is a leucine-rich PG which is found in most tissues of the body with specific localisation to fibrillar collagen similar in structure to lymph node capsule [22].

Heparin is a specialised product of mast cells [23], which can reside in both the mucosa or connective tissue component of tissue, and it is released on degranulation of mast cells. Lymph nodes normally have a large mast cell population, and more recently a new subpopulation has been identified in the

lymphatic sinuses [24]. The identification of heparin in the efferent lymph is consistent with the location and proposed function of the lymphatic-sinus mast cell (LMSC) which is thought to interact with macrophages and lymphoid cells vital in the immune response [24], therefore as the lymph bathed LMSC contacted other cells of the immune system they may have de-granulated resulting in the release of heparin.

Heparan sulphate is synthesised by most cells and is associated primarily with the plasma and basement membranes [25]. It has been demonstrated that mouse mammary cells produce two distinct heparan sulphates, a cell surface PG that is shed into the fluid environment and a basement membrane PG which accumulates beneath cells [26]. A distinct pattern of HS-PG distribution became evident, where the cell-associated PG of the perfused nodes and lymph node cultures was solely HS-PG. This is consistent with the foregoing comments on the distribution of HS-PG. A low buoyant density HS-PG of 2.8×10^4 D was consistently synthesised by the node *in vitro*, which co-purified with HA at density 1.44–1.50 g/ml. A HS-PG of equivalent Mw is produced by vascular endothelial cells in culture [27], where its suggested function is cell attachment, a function which is clearly applicable in the lymph node. The identification of a high molecular weight HS-PG (6.6 to 9.4×10^5) in both the conditioned medium and lymph node tissue is similar to HS-PG isolated from colon carcinoma [28] where it was considered an integral membrane component, possibly involved in cell adhesion.

An outstanding feature of HA synthesised and released into the growth medium of the cultured lymph node tissue was its unusually low Mw compared with that produced by most kinds of cell culture. This was especially obvious in the 5 h cultures, where there were no polymers in the high range. This phenomenon made the distinction of HA from the naturally smaller GAG difficult, but there was no doubt that much of the HA itself consisted of small polymers. In view of the intrinsic capacity of the lymph node to degrade HA, the finding of 4- and 6-saccharides strongly implicates post-secretory degradation within the node. The recovery of labeled GlcNAc in the tissue extracts suggests that there may be concurrent degradation of endogenous GAG, but is inconclusive in the absence of labeled GlcNAc-6P. Even so, the generation of such products is necessarily intracellular, since it requires phosphorylation and the action of a specific deacetylase within the cell. There is a wide variety of cells that can potentially synthesise HA in the lymph node, and it may be that in contrast with the more widely studied fibroblast and epidermal cell types, these naturally secrete small polymers. Alternatively, HA might be degraded by the free radical activity associated with the phagocytic cell types of the node or by a form of hyaluronidase active in extracellular fluids, a possibility that has not yet been examined. Either of these might explain the finding of HA oligiosaccharides in the conditioned culture medium.

At this point, it has been established that the lymph node can synthesise and release into the outflowing lymph HA as well as CS/DS-PG and heparin and that much of the HA is of low Mw, though its quantitative contribution to the observation that led to this study remains uncertain. The production of HA within the lymph nodes has an added significance, since there is increasing evidence of its participation in cellular immune responses, including cellular stimulation and cell-cell or cell-stroma adhesion. HA can also enhance phagocytosis [29,30] and pinocytosis [31], essential roles of lymphatic macrophages. This effect might also extend to the related antigen-presenting cells. Future work arising from the present study might include fractionation and identification of the lymph node cells that synthesise HA and examine in the same way the question of its partial post-synthetic degradation, and its participation in the reinforcement of lymphocyte interactions involved in immune reactions [32,33].

Acknowledgements

The authors would like to thank Professor Wayne Comper for his review of this manuscript and for his invaluable advice.

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Received 14 August 2000, revised 1 March 2001, accepted 7 March 2001