Receptors on lymphocytes for endogenous splenic glycosaminoglycans

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SUMMARY

Previous studies have shown that lymphocytes carry cell surface receptors for sulphated polysaccharides (SPS), and SPS recognition may play a role in lymphocyte migration and positioning *in vivo*. This paper describes attempts to isolate and characterize the endogenous glycosaminoglycans (GAGs) of murine spleen and determine whether splenic lymphocytes carry cell surface receptors for these GAGs. A procedure was devised for isolating GAGs from murine spleen in good yield and high purity and the GAG preparation was then radiolabelled for subsequent binding studies. It was found that the splenic GAGs bound to murine splenocytes in a saturable, rapid and reversible manner with only a small subpopulation of the splenic GAG preparation being involved in binding. This reactive species was chondroitinase ABC-resistant and nitrous acid-sensitive, indicative of a heparan sulphate/heparin-like molecule. Furthermore, using immunofluorescent flow cytometry studies it was demonstrated that the majority of spleen cells have receptors for these GAGs. Subsequent ion-exchange fractionation and SDS–PAGE analysis of chondroitinase ABC-resistant GAGs confirmed that the splenic GAG recognized by splenocytes was a heparan sulphate/heparin molecule of approximately 20,000 MW with a binding affinity to splenocytes of approximately 5×10^{-8} M. Additional binding inhibition studies indicated two possible binding sites for splenic GAGs on the splenocyte surface, one being fully inhibited by a range of SPS such as heparin (both coagulant and anticoagulant forms), pentosan sulphate, fucoidan, dextran sulphate, λ- and ι-carrageenan, and the second being partially inhibited by κ-carrageenan. The possible relevance of these heparan sulphate/heparin receptors on splenocytes to lymphocyte positioning *in vivo* is discussed.

INTRODUCTION

The molecular and cellular basis of lymphocyte migration and subsequent positioning within lymphoid tissues is not well understood. Lymphocytes exhibit defined migration patterns, showing preferences for certain lymphoid tissues (Butcher, Scollay & Weissman, 1980; Stevens, Weissman & Butcher, 1982; Chin & Hay, 1984) and sites within these tissues (De Sousa, 1981). Specific cellular interactions have been implicated in this phenomenon, the most notable being the binding of lymphocytes to high endothelial venules in lymph nodes (Butcher, Scollay & Weissman, 1979, 1980; Chin, Carey & Woodruff, 1982; Gallatin, Weissman & Butcher, 1983; Woodruff & Clarke, 1987), the reaction of lymphocytes with macrophages in the marginal zones of the spleen (Humphrey, 1980) and the interaction of lymphocytes with interdigitating cells in the T-dependent regions of lymphoid organs (Inaba & Steinman, 1986).

Recent studies in a range of biological systems have suggested that sulphated polysaccharide (SPS) recognition plays a key role in a range of cell-adhesion systems such as sponge cell aggregation (Coombe, Jakobsen & Parish, 1987), neuronal cell–cell adhesion (Cole, Loewy & Glaser, 1986), sperm-egg adhesion (Bolwell, Callow & Evans, 1980; Ahuya, 1982; Glabe *et al.*, 1982) and embryogenesis (Wenzl & Sumper, 1981; Yamaguchi & Kinoshita, 1985; Tucker, 1986). Furthermore, receptors for SPS are present on many cell types including macrophages (Bleiberg, MacGregor & Aronson, 1983; Chong & Parish, 1986), endothelial cells (Glimelius, Busch & Höök, 1978; Glabe, Yednock & Rosén, 1983) and lymphocytes (Parish, Rylatt & Snowden, 1984; Parish & Snowden, 1985; Thurn & Underhill, 1986; Parish, Hogarth & McKenzie, 1988). Thus it seems possible that SPS recognition may play a role in the migration and positioning of lymphocytes within lymphoid tissues.

Some experimental data, in fact, supports this view. It has been known for many years that certain SPS, such as heparin and dextran sulphate, cause leucocytosis and inhibit lymphocyte recirculation (Jansen *et al.*, 1962; Sasaki & Suchi, 1967; Bradfield & Born, 1974). Furthermore, fucoidan, a sulphated

Abbreviations: CPC, cetylpyridinium chloride; DDW, double distilled water; GAGs, glycosaminoglycans; PBS, phosphate-buffered saline; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; SPS, sulphated polysaccharides; TCA, trichloroacetic acid.

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polymer of L-fucose, can inhibit the binding of lymphocytes to high endothelial venules *in vitro* (Stoolman & Rosén, 1983) and it has been shown that these endothelial cells rapidly incorporate radiolabelled sulphate into a sulphated glycolipid which is secreted and causes lymphocytes to localize at intradermal sites of injection (Andrews, Milsom & Ford, 1982; Andrews, Milsom & Stoddart, 1983). Different SPS can also selectively modify entry and positioning of lymphocyte subpopulations within lymphoid organs (Brenan & Parish, 1986), possibly by blocking receptors on lymphocytes for SPS.

If SPS recognition plays such a key role in lymphocyte migration and positioning, one would predict that endogenous SPS isolated from lymphoid tissue should bind to lymphocytes and in some way effect their recirculation behaviour. This paper describes attempts to isolate and characterize the endogenous sulphated glycosaminoglycans (GAGs) present in murine spleen and determine whether murine splenic lymphocytes carry cell surface receptors for any of these GAGs.

MATERIALS AND METHODS

Mice

All mice were bred at the John Curtin School of Medical Research. C57BL/6 mice of either sex were used from 6 to 20 weeks of age.

Polysaccharides

All polysaccharides were obtained from Sigma Chemical Co. (St Louis, MO), with the exception of dextran sulphate (MW 500,000) which was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). The separation of bovine lung heparin into fractions with high and low affinity for antithrombin III was performed using an antithrombin III-coupled Sepharose column, according to the method of Parish *et al.* (1987). The polysaccharides were dissolved in 0·15 M NaCl at stock concentrations of 20 mg/ml or, in the case of hyaluronic acid and the carrageenans (because of their viscosity in solution), at 10 and 2 mg/ml, respectively, and stored at $-20°$. They were boiled for 1 min before use in binding specificity experiments.

Preparation of lymphocyte suspensions

Single cell suspensions from C57BL/6 spleen were prepared in phosphate-buffered saline (PBS) containing 0·1% (w/v) bovine serum albumin (BSA) (Fraction V, Armour Pharmaceuticals, Eastbourne, England) as described by Parish *et al.* (1974). Suspensions were depleted of red and dead cells by Isopaque–Ficoll separation (Davidson & Parish, 1975).

Isolation of splenic-GAGs

Murine C57BL/6 spleens (about 600–1200) were collected and stored frozen in PBS. Initially spleens were defrosted and drained of PBS and the packed volume measured. Half a volume of ice-cold DDW (double distilled water) was added and the mixture blended on ice in an Ato-mix blender (MSE, London, U.K.) on the maximum setting for 1 min. Blended spleens were added gradually to 10 vol of ice-cold acetone with vigorous stirring, the precipitate allowed to settle on ice for 15 min and the acetone decanted. Another 3 vol of ice-cold acetone were added, the mixture stirred vigorously for 5 min and the precipitate allowed to settle for 15 min. The final acetone wash was repeated and the acetone removed by filtration using a large Buchner funnel, under suction. The precipitate was then transfered to a glass tray and spread out to dry overnight (with occasional stirring) at room temperature in a fume hood.

The dried precipitate was weighed and ground with a mortar and pestle to a fine powder. The powder was suspended in 0·05 M Tris-HCl buffer, pH 7·5, containing 0·1% sodium azide (10 ml buffer/g of powder), pronase (15 mg/g powder, protease from *Streptomyces griseus*; Sigma Chemical Co.) added and the mixture incubated at 37° for 3 days with occasional shaking.

Following pronase treatment, undigested material was pelleted by centrifugation (2500 ***g***, 10 min), the supernatant harvested and adjusted to 0·5 M NaCl by addition of 2 M NaCl and GAGs isolated by 5% (w/v) CPC (cetylpyridinium chloride, Stansens, Kingsgrove) and ethanol precipitation as previously described (Rodén *et al.*, 1972). The ethanol-precipitated material was redissolved in DDW and contaminating DNA and peptides precipitated overnight at 4° by the addition of 0·2 vol of ice-cold 50% (w/v) trichloroacetic acid (TCA).

The precipitate was pelleted by centrifugation (16,000 ***g***, 15 min, 4°) and the supernatant dialysed for 2 hr against DDW, the dialysate being changed every 30 min. The dialysed solution was adjusted to 0·05 M Tris-HCl buffer, pH 8·0, and 0·15 M NaCl and the GAGs reprecipitated by CPC and ethanol (Rodén *et al.*, 1972). The final GAG precipitate was dissolved in DDW and the amount of sulphated polysaccharide present determined by the dimethylmethylene blue assay (Farndale, Buttle & Barrett, 1986) using chondroitin-4-sulphate as a standard. The protein content of the GAG preparation was determined by the Coomassie Blue procedure (Rylatt & Parish, 1982) and the DNA/RNA content assessed by absorbance at 260/280 nm using a DMS 100 Varian spectrophotometer.

Digestion of GAGs

For chondroitinase digestions, GAGs were dissolved in 0·1 ml of 0·05 M sodium acetate, 0·05 M Tris-HCl buffer, pH 8·0, containing 1·25 U/ml of either chondroitinase ABC (from *Proteus vulgaris*, Sigma Chemical Co.) or chondroitinase AC (from *Arthrobacter aurescens*, Sigma Chemical Co.), 0·1% (w/v) sodium azide added and the solutions incubated overnight at 37°. In the case of RNase and DNase, GAGs were digested with 20,000–25,000 U/ml of enzyme for 30 min at 37° in PBS or 0·1 M NaCl, 0·1 M $MgCl_2$ and 0·01 M Tris-HCl buffer, pH 7·5, respectively. For binding experiments solutions were then boiled for 5 min to destroy the enzyme. Control digests did not contain the enzyme. For nitrous acid cleavage of radiolabelled GAGs 9 vol of 0·24 M $NaNO_2$ in 1·8 M acetic acid were added and the mixture incubated overnight at room temperature (Lindahl *et al.*, 1973). The mixture was then either dialysed against DDW (2 hr, room temperature, with 30 min changes in DDW), lyophilized and redissolved in SDS–PAGE sample buffer (Laemmli, 1970) or dialysed against PBS if the preparation was radiolabelled and to be used for subsequent splenocyte binding assays.

Chondroitinase digestion of unlabelled GAGs was monitored by a previously described dimethylmethylene blue assay (Farndale *et al.*, 1986), although decrease in absorbance at 600 nm was used to quantify GAGs rather than increase in absorbance at 525 nm as reported earlier. The method was automated and miniaturized by performing the assays in 96-well flat-bottomed microtitre plates (Nunclon Delta, Roskilde, Denmark) and reading absorbance values on a Dynatech

MR600 microplate reader (Dynatech Labs, Alexandria, VA). Digestion of the ^{125}I-labelled GAG preparation by chondroitinases was monitored by passage of the digested sample through a PD10 column (Pharmacia) and measuring ^{125}I-labelled undigested material in the excluded peak. This elution profile was compared with that obtained with an untreated sample of labelled GAGs.

Preparation of radiolabelled GAGs

GAGs were fluoresceinated and radiolabelled with ^{125}I as described by Glabe, Harty & Rosén (1983). Unincorporated ^{125}I was removed by dialysing the radiolabelled preparations for 2 hr at room temperature against PBS.

Binding of radiolabelled GAGs to cells

Binding assays were performed in either $1{\cdot}0 \times 7{\cdot}6$ cm plastic centrifuge tubes (3DT tubes, Disposable Products, Adelaide) or, in the case of larger experiments and where timing was critical, 96-well round-bottomed microplates (Linbro, Flow Laboratories Inc., McLean, VA) were used. The reaction mixture consisted of 100 μl of ^{125}I-splenic GAGs in PBS containing 0·1% BSA to which 100 μl (4×10^6) of splenocytes were added and the cells left to react on ice for 60 min with occasional shaking. Tube assay samples were washed three times by centrifugation (300 *g*, 5 min, 4°) with 1 ml/tube of ice-cold PBS/0·1% BSA whereas in the microplate assay, cells were washed with PBS/0·1% BSA using a Titertek cell harvester (Flow Laboratories Ltd., Irvine, Renfrewshire, U.K.). Air-dried filter paper discs were collected and cell bound ^{125}I counted. To correct for non-specific binding, control binding assays were performed in the presence of 50–150-fold excess of unlabelled heparin (shown to inhibit binding of labelled splenic GAGs). In binding–inhibition experiments 50 μl aliquots of inhibitor (6·25–800 μg/ml) were placed in microplates, a constant amount of ^{125}I-splenic GAGs (50 μl, 20 μg/ml) added followed by 4×10^6 splenocytes in 100 μl and the standard binding assay performed.

Ion-exchange fractionation of GAGs

C57BL/6 splenic GAGs were prepared, iodinated (see above) and dialysed 2·5 hr against either 0·25 M NaCl, 0·01 M Tris-HCl buffer, pH 7·0 (ready for ion-exchange fractionation) or chondroitinase treatment buffer (see above). The latter sample was chondroitinase ABC digested overnight at 37° and degradation products removed by passage of the sample through a PD10 column (Pharmacia) equilibrated with 0·25 M NaCl, 0·01 M Tris-HCl buffer, pH 7·0.

A DEAE–Sepharose CL6B column ($41{\cdot}5 \times 1{\cdot}8$ cm; Pharmacia Fine Chemicals) was equilibrated with 0·25 M NaCl, 0·01 M Tris-HCl buffer, pH 7·0, the sample applied and the column washed with 50 ml of equilibration buffer prior to elution of material from the column with a 200 ml linear 0·25–1·0 M NaCl gradient, buffered with 0·01 M Tris-HCl, pH 7·0. The column flow rate was 10·2 ml/hr and 2-ml fractions were collected and assayed for NaCl content by refractometry and counted for radioactivity. Groups of fractions (20 per group) were pooled (Pools I–VII), dialysed overnight against DDW, lyophilized and redissolved in 2 ml PBS/0·1% BSA.

Biotinylation of GAGs

Splenic GAGs (2 mg in 1·0 ml DDW) were CNBr activated by the addition of 0·2 ml of CNBr (50 mg/ml in DDW) and the mixture left to react for 5 min at room temperature, the pH being maintained at 11·0 by the addition of 1 M NaOH. Unreacted CNBr was removed by passage of the splenic GAGs through a PD-10 desalting column equilibrated with 0·2 M borate buffer, pH 8·0. The excluded peak was collected (2·0 ml), 4 mg of biocytin (N-ε-biotinyl-L-lysine) added and the mixture left to react at room temperature overnight. Biotinylated GAGs were separated from free biocytin by passage through another PD-10 column and collection of the excluded peak (3·0 ml). Based on a molecular weight of 20,000, each GAG molecule was conjugated with four to five biotin molecules.

Immunofluorescent flow cytometry

Binding of biotinylated splenic GAGs to murine splenocytes was quantified by flow microfluorometry using an indirect immunofluorescence technique similar to that described by Berman & Basch (1980). Briefly, 50 μl of splenocytes (10^7/ml) were added to 50 μl of biotinylated GAGs (1·6–200 μg/ml) in PBS/0·1% BSA and the mixture incubated on ice for 60 min. Following washing in PBS/0·1% BSA the cells were incubated with a second-step avidin-fluorescein conjugate (Becton-Dickinson, Immunocytometry Systems, Mountain View, CA; 1 : 20 dilution), a third-step rabbit anti-avidin antiserum (Sigma Chemical Co.; 1 : 250 dilution) and a fourth-step fluorescein-conjugated sheep $(Fab')_2$ anti-rabbit Ig (Silenus Laboratories, Hawthorn; 1 : 10 dilution), the cells being washed twice with Eagle's minimum essential medium (F15; Gibco, Grand Island, NY)/5% fetal calf serum (FCS) between each incubation step. Labelled cells were analysed on a Becton-Dickinson FACS IV (Becton-Dickinson) with appropriate controls for each incubation step.

Gel electrophoresis

Pooled fractions of radiolabelled GAGs from the ion-exchange fractionations were analysed by one dimensional 10% and 15% SDS–PAGE according to the method of Laemmli (1970). Gels were stained with Coomassie blue, destained to localize added molecular weight markers (Bio-Rad Laboratories, Richmond, CA), then dried for autoradiography and molecular weight markers identified with radioactive ($^{35}SO_4^{2-}$) ink. Dried gels were autoradiographed at −70° on Kodak XAR-5 film using Cronex intensifying screens.

RESULTS

Isolation of splenic-GAGs

Earlier studies have shown that murine lymphocytes carry cell surface receptors for sulphated polysaccharides (Parish *et al.*, 1984; Parish & Snowden, 1985; Thurn & Underhill, 1986; Parish *et al.*, 1988). To determine if there are endogenous sulphated polysaccharides in murine spleen that bind to murine splenic lymphocytes, GAGs were first isolated from murine spleens. Briefly, homogenized spleens were delipidated by acetone extraction, deproteinized and GAG side-chains released by pronase digestion and then sulphated-GAGs precipitated by CPC. A high NaCl concentration (0·5 M) was used during CPC precipitation to ensure that only sulphated GAGs were precipitated (i.e. no hyaluronic acid contaminated the preparation) and to minimize DNA contamination. TCA precipitation was then used to remove residual contaminating DNA and peptides and,

Table 1. Effect of different degradative procedures on binding of splenic GAGs to splenic lymphocytes

Treatment	% degradation: Unlabelled GAGs*	% degradation: Labelled GAGs†	% binding of labelled GAGs to splenocytes‡
Nil	—	—	100±3·0
Chondroitinase ABC	84·5±16	60·3	130±4·3
Chondroitinase AC	60·9±22	18·4	96±3·7
Nitrous acid	< 15	30·9	56±3·7

* Degradation of unlabelled GAGs monitored by a dimethylmethylene blue assay. Each data point is the mean of three replicates ± SE.

† Represents ^{125}I-label associated with undegraded GAGs (see the Materials and Methods). Each data point represents a single determination.

‡ Mean of three to six data points ± SE using 10 μg/ml of labelled GAGs.

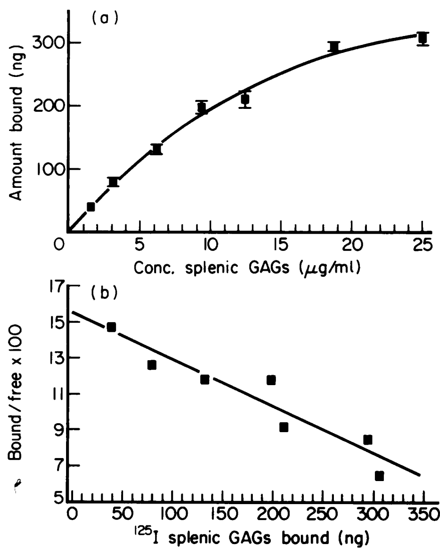

Figure 1. Analysis of binding of radiolabelled murine splenic GAGs to murine splenocytes. (a) Concentration dependence of binding of ^{125}I-splenic GAGs to splenocytes. The data shown have been corrected for non-specific binding as defined by the amount of radioactivity associated with the splenocytes in the presence of a 50-fold excess of unlabelled heparin. Washing of cells was performed by centrifugation. The data shown are the mean of five replicates ± standard errors of means. (b) Scatchard analysis of splenic GAG binding data (correlation coefficient = 0·89).

finally, the 'crude' splenic GAGs were precipitated with CPC and ethanol (Rodén *et al.*, 1972). With C57BL/6 mice, 1000 spleens produced 21 g of acetone extracted powder which yielded 6·5 mg of 'crude' splenic GAGs. Based on protein and DNA/RNA assays, the GAG preparation contained no detectable protein and contained less than 1% RNA and DNA.

The GAG composition of the 'crude' splenic GAG preparation was assessed by chondroitinase ABC and AC digestion

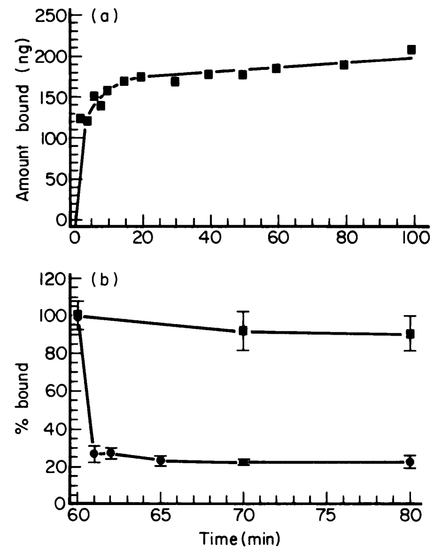

Figure 2. Analysis of binding of radiolabelled murine splenic GAGs to splenocytes. (a) Time-course of binding of ^{125}I-splenic GAGs to splenocytes. Data was corrected for non-specific binding as in Fig. 1 and each data point is the mean of two replicates. (b) Reversibility of binding of ^{125}I-splenic GAGs to splenocytes in the presence of either no competitor (■) or a 150-fold excess of unlabelled heparin (●). Each data point is the mean of three replicates ± SEM. All washing of cells was performed on the harvester.

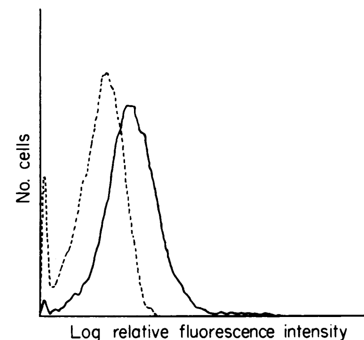

Figure 3. Ability of biotinylated splenic GAGs at 100 μg/ml (——) to bind to murine splenocytes as quantified by flow microfluorometry. Control profile (- - -) representative of cells preincubated with second, third and fourth stage reagents.

and nitrous acid treatment. It was found that 84·5 ± 16% of the preparation was chondroitinase ABC-sensitive and 60·9 ± 22% chondroitinase AC sensitive (Table 1). Nitrous acid treatment, which selectively degrades heparan sulphate/heparin chains, destroyed < 15% of the 'crude' splenic GAG preparation. Since fluoresceinated and ^{125}I-labelled splenic GAGs were used in subsequent binding studies (see below) it was important to determine the composition of the labelled GAG preparation. It was found (Table 1) that the majority of the labelled GAGs were chondroitinase ABC-sensitive although substantially less was chondroitinase AC-sensitive compared with the unlabelled material. This suggests that dermatan sulphate in the prep-

Table 2. Polysaccharide inhibition of binding of C57BL/6 splenic GAGs to C57BL/6 splenic lymphocytes

Polysaccharide	Major monosaccharides present	Moles/disaccharide Carboxyl groups	Sulphate groups	Mean percentage control binding ± SE
Nil	—	—	—	100±5·4
Hyaluronic acid	GlcUA GlcNAc	1·0	0	84·1±1·9
Chondroitin-4-sulphate	GlcUA GalNAc	1·0	1·0	97·9±2·2
Chondroitin-6-sulphate	GlcUA GalNAc	1·0	1·0	101±1·0
Dermatan sulphate	IdUA GalNAc GlcUA	1·0	1·0–2·0	96·0±1·5
Keratan sulphate	Gal GlcNAc	0	1·0–2·0	89·7±4·7
Heparan sulphate	IdUA GlcNAc GlcUA	1·0	1·0–2·0	82·1±6·1
Fucoidan	L-Fuc	0	2·0	12·7±1·0
Pentosan sulphate	D-Xyl	0	4·0	12·1±1·0
Dextran sulphate	D-Glc	0	4·6	18·0±0·8
κ-carrageenan	D-Gal	0	1·0	46·7±1·9
λ-carrageenan	D-Gal	0	3·0	18·9±0·5
ι-carrageenan	D-Gal	0	2·0	28·0±1·0
Heparin	IdUA GlcNAc GlcUA	1·0	2·0–3·0	17·9±1·3
Heparin, high affinity*	IdUA GlcNAc GlcUA	1·0	2·0–3·0	16·0±1·4
Heparin, low affinity*	IdUA GlcNAc GlcUA	1·0	2·0–3·0	19·3±0·9

Each percentage is the mean of three data points.
* Heparin separated into fractions with high and low affinity for antithrombin III.

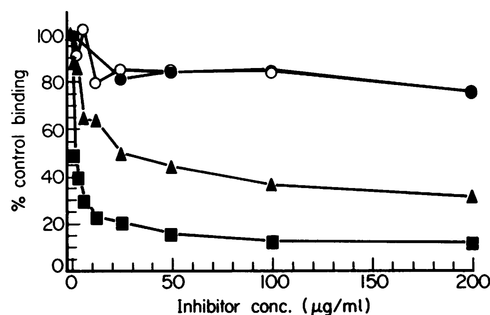

Figure 4. Ability of varying concentrations of unlabelled hyaluronic acid (○), chondroitin 4-sulphate (●), κ-carrageenan (▲) and heparin (■) to inhibit binding of ^{125}I-splenic GAGs to splenocytes. Results are expressed as the percentage of splenic GAGs binding in the absence of inhibitor. Washing of cells was performed on the cell harvester. Each data point is the mean of two replicates.

aration may have been more efficiently fluoresceinated than the chondroitin sulphates. Similarly, there appeared to be a somewhat higher proportion of nitrous acid sensitive GAGs in the labelled preparation.

Binding of splenic GAGs to splenocytes

The isolated splenic GAGs were radiolabelled with ^{125}I and their binding to C57BL/6 splenocytes assessed. Figure 1a depicts a typical binding curve that is indicative of saturable binding. The

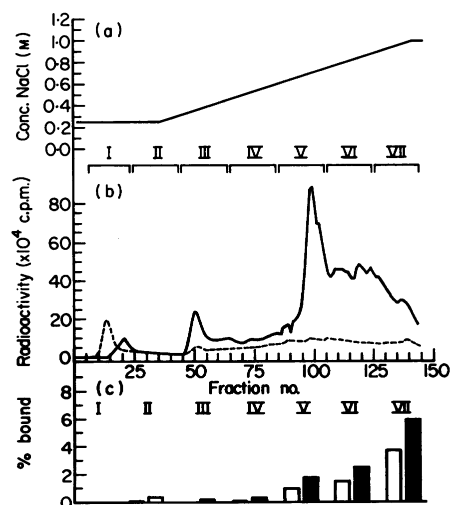

Figure 5. A ^{125}I-splenic GAG preparation, either untreated or digested with chondroitinase ABC, was applied to a DEAE–Sepharose CL6B column and eluted with a 0·25–1·0 M NaCl gradient in 10 mM Tris-HCl buffer, pH 7·0. (a) Salt gradient generated during column run. (b) Radioactivity detected in different column fractions with untreated (——) or chondroitinase ABC digested (– – –) splenic-GAGs. Fractions were collected into Pools I–VII as indicated. (c) Ability of radioactive material in Pools I–VII to bind to murine splenocytes. The percent bound for untreated (□) and chondroitinase ABC treated (■) splenic-GAGS is given. Washing of cells was performed by centrifugation.

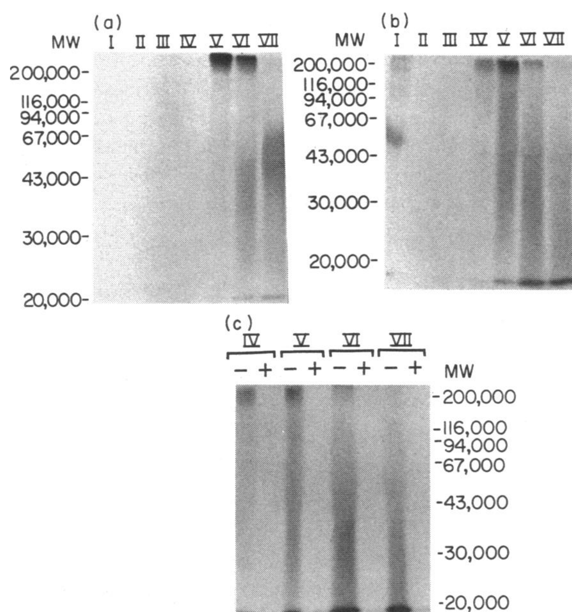

Figure 6. SDS-PAGE analysis of ^{125}I-splenic GAG pools isolated by ion-exchange chromatography (see Fig. 5). (a) Pools I–VII from untreated splenic GAGs. Exposure time 24 hr. (b) Pools I–VII from chondroitinase ABC-treated splenic GAGs. Exposure time 7 days. (c) Pools IV–VII from chondroitinase ABC-treated splenic GAG which were either nitrous acid digested (+) or untreated (−). Exposure time 7 days. The position of molecular weight markers is indicated.

data presented are corrected for non-specific binding by the inclusion of a 50-fold excess of unlabelled heparin as binding-inhibition experiments (see later) showed that heparin was a potent inhibitor of splenic GAG binding. At all concentrations non-specific binding represented less than 15% of specific binding. The data when applied to a Scatchard plot (Fig. 1b) approached linearity (correlation coefficient 0·89) with 138 ± 15 ng of splenic GAGs bound/10^6 cell. Subsequent kinetic studies demonstrated that the majority of binding occurred very rapidly (< 5 min incubation) and most binding had occurred after 15 min of incubation (Fig. 2a). Furthermore, bound GAGs were rapidly and effectively displaced by a 150-fold excess of unlabelled heparin (Fig. 2b).

At this stage it was important to determine whether all splenocytes or a distinct subpopulation were involved in splenic GAG binding. In order to resolve this issue, splenocytes were reacted with biotinylated-GAGs and bound GAGs detected by immunofluorescent flow cytometry using an amplification procedure involving fluoresceinated avidin, rabbit anti-avidin antibodies and fluoresceinated sheep anti-rabbit Ig. Typical flow cytometry profiles are depicted in Fig. 3 and it is evident that, when compared with control binding, the majority of spleen cells bound the splenic GAGs, although there may be a very small subpopulation of cells (< 2%) which exhibit high density binding. Flow cytometry profiles revealed splenic GAG binding at concentrations ranging from 0·8 to 100 μg/ml, data consistent with the binding assay depicted in Fig. 1a.

Polysaccharide specificity of splenic GAG binding

A range of different polysaccharides were tested for their ability to inhibit the binding of ^{125}I-splenic GAGs to spleen cells. The inhibitory activity of these different polysaccharides and their relevant structural features are presented in Table 2. Splenic GAG binding was almost completely inhibited (approximately 80%) by heparin, pentosan sulphate, fucoidan, dextran sulphate, λ- and ι-carrageenan and partially inhibited (approximately 50%) by κ-carrageenan. Hyaluronic acid, chondroitin-4- and -6-sulphate, dermatan sulphate, keratan sulphate and heparan sulphate (bovine kidney) had little or no effect on GAG binding. Figure 4 depicts the ability of hyaluronic acid, chondroitin-4-sulphate, κ-carrageenan and heparin at a range of concentrations (1·6–200 μg/ml) to inhibit splenic GAG binding. Heparin was a potent inhibitor of GAG binding, inhibiting binding by 85–90% at high concentrations. Furthermore, heparin fractions with either high or low affinity for antithrombin III were equally effective at inhibiting GAG binding. In contrast, hyaluronic acid and chondroitin-4-sulphate had little effect on GAG binding even at the highest concentrations tested whereas κ-carrageenan exhibited an intermediate inhibition profile, partially inhibiting (50–60%) GAG binding to murine spleen cells.

Characterization of splenic GAGs which bind to splenocytes

Chondroitinase digestion of the splenic-GAG preparation demonstrated that the majority of the GAGs belonged to the chondroitin sulphate family (Table 1). It was important, therefore, to determine whether chondroitinase ABC and AC digestion had any effect on the binding of splenic GAGs to murine splenocytes. It was found (Table 1) that chondroitinase AC digestion had no effect on GAG binding (i.e. $96 \pm 3{\cdot}7\%$ of control binding following enzyme treatment), whereas chondroitinase ABC digestion actually resulted in a significant increase in binding (i.e. $130 \pm 4{\cdot}3\%$ of control binding following digestion). Similarly RNase and DNase digestion of the GAGs had no effect on binding (data not shown). In contrast, nitrous acid treatment resulted in a substantial reduction in GAG binding (i.e. $56 \pm 3{\cdot}7\%$ of control binding following treatment). These data indicate that a subpopulation of splenic GAGs interacts with murine spleen cells and the relevant GAGs were not chondroitin sulphates but probably belong to the heparan sulphate/heparin family.

To further characterize the chondroitinase ABC-resistant GAGs the radiolabelled splenic GAG preparation was chondroitinase ABC-digested prior to application and fractionation on a DEAE–Sepharose column. The fractionation profile was compared with that obtained for undigested splenic GAGs (Fig. 5). With the undigested GAG preparation the bulk of the radioactivity was eluted at > 0·6 M NaCl. As was previously observed a large proportion of the splenic GAG preparation was destroyed by chondroitinase ABC digestion (Fig. 5b). However, the remaining radioactivity was eluted from the DEAE–Sepharose column over a wide range of NaCl concentrations. Column fractions were then screened for their content of GAGs which bound to splenocytes (Fig. 5c). In order to expedite the screening procedure column fractions were collected into seven pools (Pools I–VII) each containing 20 fractions. Pools I–IV from both column runs contained little or no splenic GAGs which bound to splenocytes. In contrast, Pools V, VI and VII contained material (eluted at > 0·6 M NaCl) which bound to murine splenocytes, Pool VII (> 0·9 M NaCl) containing the most activity and enrichment of binding activity

occurring in pools from the chondroitinase ABC-digested preparation.

To characterize further the splenic GAGs present in the eluted material, Pools I–VII of both untreated and chondroitinase ABC-treated samples were analysed by SDS–PAGE. In Pools V–VII from both untreated and chondroitinase-treated samples, radiolabelled material was detected throughout the gel, a pattern characteristic of the polydispersed nature of GAGs (Fig. 6a and b). Nevertheless, chondroitinase digestion tended to destroy the high molecular weight material (> 200,000 MW) and enriched for low molecular weight GAG side-chains close to the dye front, particularly in Pools VI and VII. Subsequent nitrous acid treatment (Fig. 6c) resulted in total elimination of the chondroitinase resistant material in Pools IV–VII indicating that the residual GAGs were heparan sulphate/heparin in nature. Additional SDS–PAGE runs on 15% gels revealed that the mean molecular weight of chondroitinase ABC resistant GAGs in Pools VI and VII was approximately 20,000 (range 10,000–50,000 MW) (data not shown). It should be emphasized that this estimate is somewhat arbitrary due to the use of protein rather than defined polysaccharide standards when determining the GAG molecular weights.

DISCUSSION

This paper describes attempts to isolate and characterize sulphated GAGs from murine spleen which could be involved in controlling the migration and positioning of murine splenic lymphocytes. A procedure was devised for isolating sulphated GAGs from murine spleen in good yield and high purity and the resultant GAG preparation was used in subsequent binding studies. It was found that isolated splenic GAGs bound to murine splenocytes in a saturable (Fig. 1a), rapid (Fig. 2a) and reversible (Fig. 2b) manner, characteristics consistent with a cell surface receptor of high affinity and specificity. Further analysis of the splenic GAG preparation revealed that a small subpopulation of the GAG molecules bound to spleen cells. The reactive GAGs were chondroitinase ABC resistant and nitrous acid sensitive, properties consistent with polysaccharides belonging to the heparan sulphate/heparin family. Furthermore, immunofluorescent flow cytometry studies demonstrated that the majority of murine splenic lymphocytes express binding sites for the relevant splenic GAGs.

It should be noted that nitrous acid treatment of the ‘crude’ splenic GAG preparation reduced binding to spleen cells by only 44% (Table 1). However, subsequent studies revealed that GAGs present in DEAE column fractions highly enriched for binding activity were entirely nitrous acid sensitive as judged by SDS–PAGE analysis (Fig. 6). This discrepancy may be due to nitrous acid producing small fragments (for example tetra/pentasaccharides) which are not detected by SDS–PAGE but still retain some binding activity.

Based on the data presented an estimate can be made of the binding affinity of the lymphocyte receptors for splenic GAGs. Since probably only 15% of the splenic GAG preparation was involved in binding, and assuming a mean molecular weight of the GAGs of 20,000, the binding sites on spleen cells have an apparent affinity constant of 5×10^{-8} M. This affinity is 50-fold higher than the affinity of the interaction between heparin and antithrombin III (Lindahl & Höök, 1978).

Some interesting features of the splenocyte/splenic GAG interaction were revealed by the binding inhibition studies (Table 2, Fig. 4). First, out of 13 polysaccharides tested, only seven (heparin, fucoidan, pentosan sulphate, κ-, λ- and ι-carrageenan) inhibited binding (Table 2). Charge density alone can not account for the binding inhibition observed as chondroitin-4- and -6-sulphate and dermatan sulphate have a higher charge density than κ-carrageenan and yet only the latter polysaccharide inhibited binding. Similarly ι-carrageenan and fucoidan inhibited binding, whilst the chondroitin sulphates (of similar charge density) did not. Second, minor changes in the sulphation pattern of the polysaccharides had substantial effects on inhibitory activity as κ- and ι-carrageenan, which differ only in the number of sulphates per disaccharide (one and two, respectively), showed significant differences in their ability to inhibit GAG binding. Third, the observation that κ-carrageenan only partially inhibited splenic GAG binding suggests that splenocytes may contain more than one type of binding site on their cell surface, one site not reacting with κ-carrageenan and the other site reacting with all seven inhibitory polysaccharides. Similarly, diversity of binding sites for SPS has been detected on murine thymocytes (Parish *et al.*, 1988). Fourth, heparin fractions with either high or low affinity for antithrombin III exhibited similar inhibitory activity indicating that the antithrombin III binding site on heparin plays little or no role in splenocyte binding.

Although the splenic GAGs which bind to lymphocytes appear to belong to the heparan sulphate/heparin family it is interesting to note that heparan sulphate isolated from bovine kidney did not inhibit splenic GAG binding. This observation is not particularly surprising as heparan sulphates exhibit great structural diversity, earlier studies suggesting that theoretically 10^{36} types of heparan sulphate could occur in animal tissues (Dietrich, Nader & Straus, 1983). Presumably, therefore, bovine kidney heparan sulphate, which is less sulphated than heparin, does not contain the correct positioning of sulphates for binding to occur. In this context, cell surface receptors which can distinguish subtle changes in the structure of sulphated polysaccharides have been detected on a range of cell types such as lymphocytes (Parish & Snowden, 1985; Parish *et al.*, 1988), macrophages (Chong & Parish, 1986), endothelial cells (Glabe *et al.*, 1983) and neurones (Vidovic *et al.*, 1986).

Finally, it should be emphasized that although the present study has demonstrated that splenic lymphocytes express cell surface receptors for splenic GAGs, the functional significance of this interaction remains to be determined. An attractive hypothesis is that splenic heparan sulphates/heparin in some way direct the migration and positioning of lymphocytes within the spleen (Brenan & Parish, 1986). Experiments are underway in order to test this hypothesis.

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