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Characterization of lymphocyte receptors for glycosaminoglycans

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SUMMARY

This paper describes attempts to isolate and characterize glycosaminoglycan (GAG)-binding molecules on the surface of lymphocytes and lymphoma cell lines and relate their expression to splenic and lymph node homing capacity. Initial binding studies with radiolabelled GAG and rosetting studies with GAG-coupled erythrocytes revealed that there are receptors on lymphocytes for the major classes of GAG (i.e. hyaluronic acid, chrondroitin sulfates, heparin), but lymphocytes bind heparin much more avidly than other GAG species. Analysis of the binding of solubilized radiolabelled cell-surface molecules to immobilized GAG revealed cell-type specific expression of GAG-binding molecules. Thus, each of four lymphoma cell lines tested gave a characteristic pattern of GAG-binding molecules, some molecules being unique to a particular cell line and others being shared by some of the lines. Similarly, splenocytes expressed at least 10 distinct GAG-binding molecules with molecular weights (MW) ranging from 10,000 to 100,000, whereas thymocytes expressed additional GAG-binding proteins of 190,000 and 250,000 MW. Furthermore, splenocytes differed from thymocytes by possessing a unique family of cell-surface molecules which reacted with each GAG. Immunoprecipitation studies demonstrated that the GAG-binding molecules on splenocytes did not correspond to any of the cell-surface antigens tested, notably the cell adhesion molecules MEL-14, CD11/CD18 and CD44, although CD8 bound weakly to heparin. Four lymphoma cell lines with well-characterized migration properties were examined for GAG-binding molecules which may control lymphocyte migration. It was found that no one GAG-binding protein could be correlated with the entry of cells into a particular lymphoid organ. Nevertheless, the role of GAG-binding molecules in the subsequent positioning of lymphocytes within lymphoid organs requires further investigation.

INTRODUCTION

In recent years many cell-surface antigens on lymphocytes have been identified and characterized. Some of these molecules, such as MEL-14 and CD44, have been implicated in lymphocyte migration and, in particular, appear to play a role in the interaction of lymphocytes with high endothelial venules (HEV).[1,2] However, despite such findings, the endogenous ligands for many of these molecules have not been identified. In this context, a large body of data suggests that carbohydrate recognition may play a role in many forms of lymphocyte adhesion and migration. For example, sulphated and phosphorylated carbohydrates inhibit the lymphocyte/HEV interaction *in vitro*[3] and the lymphocyte homing receptor MEL-14 is probably a phosphomannosyl-specific lectin.[2,4,5] Furthermore, *in vivo* studies have shown that sulphated polysaccharides can induce leucocytosis and can selectively effect the entry and subsequent positioning of lymphocytes within lymphoid organs.

Recent studies from this laboratory have shown that murine splenocytes interact with endogenous splenic glycosaminoglycans (GAG) in a highly avid and specific manner.[10] These observations raised the possibility that cell-surface receptors on splenic lymphocytes for splenic GAG may direct the migration and positioning of lymphocytes within the spleen. In order to examine such a possibility this paper describes attempts to identify and characterize GAG-binding molecules on the surface of lymphocytes and lymphoma cell lines and relate their expression to splenic and lymph node homing capacity. Of particular interest was whether well-characterized cell-surface molecules, notably those implicated in lymphocyte migration, bind GAG.

Abbreviations: BSA, bovine serum albumin; CMC, carboxymethyl cellulose; GAG, glycosaminoglycan; H33342, fluorescent dye Hoechst 33342; HEV, high endothelial venule; Ig, immunoglobulin; mAb, monoclonal antibody; MLN, mesenteric lymph node; PBS, phosphate-buffered saline; PLN, peripheral lymph node; PP, Peyer's patch; SDS-PAGE: sodium dodecyl sulphate–polyacrylamide gel electrophoresis; SRBC, sheep red blood cells; TTS, 0·15 M NaCl, 0·05 M Tris-HCl, pH 8·0, 0·5% Triton X-100.

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MATERIALS AND METHODS

Mice

All mice were bred at the John Curtin School of Medical Research. C57BL/6 mice of either sex were used as a source of splenic GAG, whereas in all other experiments female mice were used from 6 to 20 weeks of age. C57BL/6, BALB/c and CBA mice were used for *in vivo* migration studies and BALB/c mice for maintaining cell lines.

Glycosaminoglycans (GAG)

Endogenous splenic GAG were isolated from C57BL/6 spleens as reported recently.[10] All other GAG were obtained from Sigma Chemical Co. (St Louis, MO). The GAG were dissolved in 0·15 M NaClat stock concentrations of 20 mg/ml, or in the case of hyaluronic acid at 10 mg/ml, and stored at −20°. They were boiled for 1 min before use.

Antibodies

The following monoclonal antibodies (mAb) against murine lymphocyte cell-surface antigens were used: anti-Thy-1 (clone T24-31.7), kindly supplied by Dr I. Trowbridge, Salk Institute, La Jolla; anti-Ly-5.1 (clone S-450-15.2), anti-ThB (clone 53-9.2.8) and anti-Ly-15.2 (clone 8-6.2), kindly provided by Professor I. McKenzie, University of Melbourne; and anti-Pgp-1 (clone IM7.8.1), anti-MEL-14, anti-Ly-2 (clone 53.6.7), anti-FcR (clone 2.4G2) and anti-CD3 (clone C363), generously supplied by Dr R. Ceredig, Australian National University. The mAb used were immobilized on one of four substrata. Purified anti-Ly-15.2 and anti-Pgp-1 antibodies were covalently coupled to CNBr-activated Sepharose-4B beads (Pharmacia, Uppsala, Sweden). A number of rat mAb (MEL-14, anti-Ly-2, anti-FcR and anti-CD3) were adsorbed to sheep anti-rat Ig-coupled (Silenus Laboratories, Hawthorn, Australia) Sepharose 4B beads. The remaining antibodies were bound to either protein A-coupled Sepharose CL-4B beads (Pharmacia) (anti-ThB and anti-Ly-5.1) or recombinant protein G-coupled agarose (Genzyme Corp, Boston, MA) (anti-Thy-1).

Cell lines

The B-cell lines $RD10_s$ (gift from Dr K. Walker, University of Sydney, Australia) and BCL.1[11] (supplied by Dr A. Ramsay, National University) were passaged continuously in BALB/c mice by injecting 10^6 $RD10_s$ cells intravenously and 2×10^6 BCL.1 cells intraperitoneally. Splenic tumours were removed from $RD10_s$ and BCL.1 injected mice 14 days and 16 days post-injection, respectively. Organs were gently teased through a fine wire mesh and single-cell suspensions were prepared for re-injection.

All other T-lymphoma lines were cultured *in vitro* in Eagle's minimum essential medium (F15; Gibco, Grand Island, NY) supplemented with 2·2 g/l sodium bicarbonate, 5–10% foetal calf serum and antibiotics (penicillin 120 mg/l, streptomycin 200 mg/l, neomycin 200 mg/l).

Preparation of cell suspensions

Single-cell suspensions from mouse lymphoid organs, liver and lung were prepared in phosphate-buffered saline (PBS) containing 0·1% (w/v) bovine serum albumin (BSA) (Fraction V; Armour Pharmaceuticals, Eastbourne, E. Sussex, U.K.), as described by Parish *et al.*[12] Spleen cell suspensions were depleted of red and dead cells by Isopaque–Ficoll separation.[13]

Blood was collected from sheep in Alsever's solution and the red cells washed four times in 20 volumes of 0·15 M NaCl by centrifugation (600 ***g*** 5 min, 20°) just before use.

Rosetting assays

For rosetting assays, polysaccharides were coupled to sheep red blood cells (SRBC) via $CrCl_3$.[14] For each GAG, SRBC coupling was optimized by using a range of $CrCl_3$ concentrations. The rosetting of murine lymphocytes with polysaccharide-coupled SRBC was carried out in 96-well round-bottomed microplates (Linbro Chemical Co., New Haven, CI), as reported previously.[14]

Binding of radiolabelled GAG to cells

GAG were fluoresceinated and radiolabelled with ^{125}I[15] and binding of radiolabelled GAG to lymphocytes was performed in 96-well microplates, as described previously, cells being collected and washed on filters using a cell harvester.[10] To correct for non-specific binding, control binding assays were performed in the presence of a 50-fold excess of unlabelled GAG.

Binding of radiolabelled cell-surface molecules to immobilized GAG

Lymphocytes and lymphoma cell lines were cell surface labelled with ^{125}I using the lactoperoxidase catalysed method[16] and detergent lysates prepared in 0·15 M NaCl, 0·05 M Tris-HCl, pH 8·0, 0·5% Triton X-100 (TTS) as described earlier.[17] In order to avoid labelling of intracellular components, cells of very high viability (>98%) were used, the cells being centrifuged on Isopaque–Ficoll[13] just prior to use. The radiolabelled lysates (0·2 ml derived from 5×10^6–10^7 cells) were then bound to 50 μl of carboxymethyl cellulose (CMC) fibres (CM-23, Whatman, Maidstone, Kent, U.K.), which had been coupled with GAG using $CrCl_3$.[17] After incubation on a rotator for 60 min at 4°, the fibres were washed five times with TTS and the final sixth wash with 0·15 M NaCl, 0·05 M Tris-HCl, pH 8·0. Bound material was then eluted either by boiling (5 min) the fibres in 50 μl of reduced sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer or by adding 2 M NaCl, 0·05 M Tris-HCl, pH 8·0, 0·5% Triton X-100 and desalting the eluate by passage through a PD-10 column (Pharmacia) equilibrated with TTS.

The unabsorbed lysate or 2 M NaCl fibre eluates were monitored for their content of different cell-surface antigens by immunoprecipitation with different mAb-coupled Sepharose 4B beads. This involved incubating the lysates/eluates (0·2–0·5 ml) with mAb-coupled beads (10–15 μl) for 18 hr at 4° on a rotator, washing beads six times with TTS and eluting bound antigen by boiling beads (5 min) in reduced SDS–PAGE sample buffer (50 μl). To reduce binding of ^{125}I-labelled mouse Ig to sheep anti-rat Ig (Silenus Laboratories, Hawthorn, Australia) coupled beads, radiolabelled lymphocyte lysates were incubated overnight with uncoupled sheep anti-rat Ig beads (0·25 ml lysate/10 μl beads). In addition, binding of radiolabelled lysates to mAb-coupled anti-rat Ig beads was performed in the presence of 1% heat-inactivated normal mouse serum.

Immunoprecipitated antigens and GAG-fibre eluates were analysed by one-dimensional SDS–PAGE on 8–18% gradient gels according to the method of Laemmli[18] and radiolabelled protein bands detected by autoradiography.

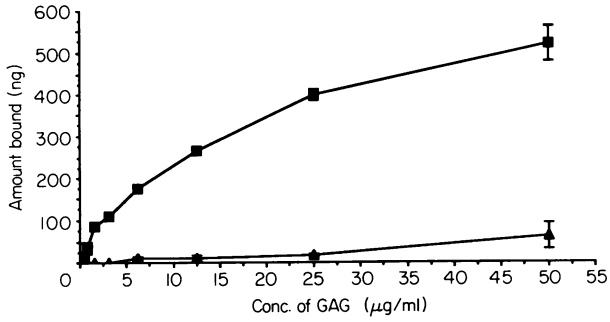

Figure 1. Analysis of binding of ^{125}I-labelled chondroitin-4-sulphate (▲) and heparin (■) to murine splenocytes. The data shown have been corrected for non-specific binding by subtracting the amount of radioactivity bound in the presence of a 50-fold excess of unlabelled GAG. Each data point is the mean of three replicates ± standard error.

Analysis of lymphocyte migration

The migration of lymphocytes and lymphoma cell lines was monitored *in vivo* by labelling cells with the fluorescent dye Hoechst 33342 (H33342; Sigma).[19,20] Recipient mice of the appropriate strain were injected intravenously with 2×10^7 H33342-labelled cells in 0·5 ml of PBS. Lymphoid organs, liver and lungs were removed from recipient mice 2–24 hr post-injection and the content of H33342-labelled cells quantified in cell suspensions of each organ by fluorescence microscopy.[19]

In the case of positioning studies, spleens were placed directly into 4% paraformaldehyde in PBS for overnight fixation. Topographical localization of fluorescent cells was visualized by a modification of the method of Brenan *et al.*[20] Fixed murine spleens were sectioned approximately in half, placed on nonfluorescent silicone rubber (Silastic, Dow Corning, Midland, MI) and viewed and photographed under a fluorescence microscope.

RESULTS

Detection of GAG-binding proteins on the surface of lymphocytes

Initially, three approaches were used to detect receptors for GAG on the surface of lymphocytes. Firstly, binding studies were performed with ^{125}I-labelled GAG, this approach giving a gross indication of whether GAG-binding molecules are present on the lymphocyte surface. Secondly, GAG-coupled erythrocytes were examined for their ability to form rosettes with murine lymphocytes; rosetting assays have the advantage that, due to multipoint binding, they are much more sensitive than binding assays with radiolabelled GAG and, furthermore, the percentage of lymphocytes carrying receptors for a particular GAG can be assessed. Thirdly, the molecular nature of the cell-surface receptors for GAG was determined by binding ^{125}I cell-surface labelled lysates of lymphocytes to immobilized GAG and characterizing bound molecules by SDS–PAGE.

A range of polysaccharides, namely, hyaluronic acid, chondroitin-4-sulphate, chondroitin-6-sulphate, dermatan sulphate, keratan sulphate, heparin and heparan sulphate, were radiolabelled with ^{125}I and their binding to splenocytes assessed. The data presented in Fig. 1 depict the specific binding of ^{125}I-labelled heparin and ^{125}I-labelled chondroitin-4-sulphate to splenocytes. The binding of heparin to splenocytes shows a typical binding curve indicative of saturable binding and is similar to that observed for the binding of endogenous splenic GAG to splenocytes.[10] A Scatchard plot of the data (not shown) indicated that the line approached linearity (correlation coefficient 0·81) with $9{\cdot}1 \times 10^6$ molecules of heparin bound to the surface of each splenocyte with a binding affinity of $1{\cdot}1 \times 10^{-6}$ M (assuming a molecular weight of 10^4 for heparin). Non-specific binding of heparin was found to be < 15% of specific binding. In contrast, there was much less binding of ^{125}I-labelled chondroitin-4-sulphate to splenocytes and specific binding was too low for Scatchard analysis. It should be emphasized, however, that the binding assay was found to be relatively insensitive due to the high non-specific binding of radiolabelled polysaccharides. For example, in the case of chondroitin-4-sulphate, it was calculated that maximum non-specific binding at 50 μg/ml was $3{\cdot}4 \times 10^5$ molecules/cell and similar non-specific binding was observed with hyaluronic acid, chondroitin-6-sulphate, dermatan sulphate, keratan sulphate and heparan sulphate. This implied that to calculate accurately the binding affinities of GAG to lymphocytes under saturating conditions, much greater than $3{\cdot}4 \times 10^5$ molecules of ^{125}I-labelled GAG would need to be bound to each cell. Thus, in order to detect relatively low numbers of GAG receptors on lymphocytes the rosetting approach was used.

Table 1. Rosetting of lymphocytes from thymus and spleen with GAG-coupled sheep erythrocytes

GAG	% Rosettes	
	Spleen	Thymus
Hyaluronic acid	62·3 ± 2·9	21·0 ± 1·5
Chondroitin-4-sulphate	62·7 ± 1·9	54·3 ± 3·2
Chondroitin-6-sulphate	72·3 ± 4·2	64·3 ± 1·2
Heparin	82·0 ± 1·7	90·3 ± 0·3

Each value is the mean of three replicates ± standard error.

Results of the rosetting of murine lymphocytes with GAG-coupled SRBC indicate between 20% and 90% of splenocytes and thymocytes express receptors for hyaluronic acid, chondroitin-4-sulphate, chondroitin-6-sulphate and heparin, although differences were seen between the two lymphocytes populations in the percentage of cells rosetting with different GAG (Table 1). These data indicate that lymphocytes express receptors for all classes of GAG, although the binding studies (Fig. 1) imply that lymphocytes more avidly bind heparin than other GAG. Earlier rosetting studies with SRBC suboptimally coupled with GAG demonstrated the preponderance of heparin recognition.[14] Inclusion of soluble GAG (50 μg/ml) in the rosetting assays completely inhibited rosette formation indicating the specificity of rosette formation.

In an attempt to characterize GAG binding molecules on the surface of lymphocytes, ^{125}I-labelled cell surface molecules were bound to a variety of immobilized GAG and bound molecules characterized by SDS–PAGE. Murine splenocytes were seen to express at least 10 distinct GAG-binding molecules with a wide range of molecular weights (i.e. approximately 10,000–20,000, 33,000, 40,000, 60,000, 90,000 and 100,000). However, each

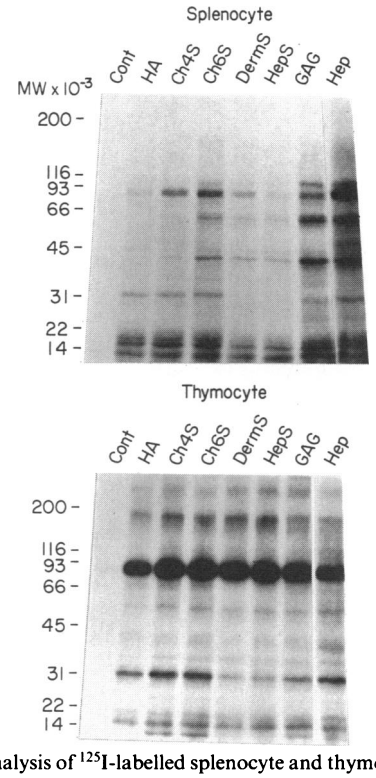

Figure 2. Analysis of ^{125}I-labelled splenocyte and thymocyte cell-surface molecules which bind to immobilized GAG. Bound molecules were run on a 8–18% gradient SDS–PAGE gel and gels were autoradiographed for 2 days except in the case of heparin-binding molecules where gels were exposed for one day. Molecular weight markers are indicated. HA, hyaluronic acid; Ch4S, chondroitin-4-sulphate; Ch6S, chondroitin-6-sulphate; DermS, dermatan sulphate; HepS, heparan sulphate; GAG, endogenous splenic-GAG; Hep, heparin.

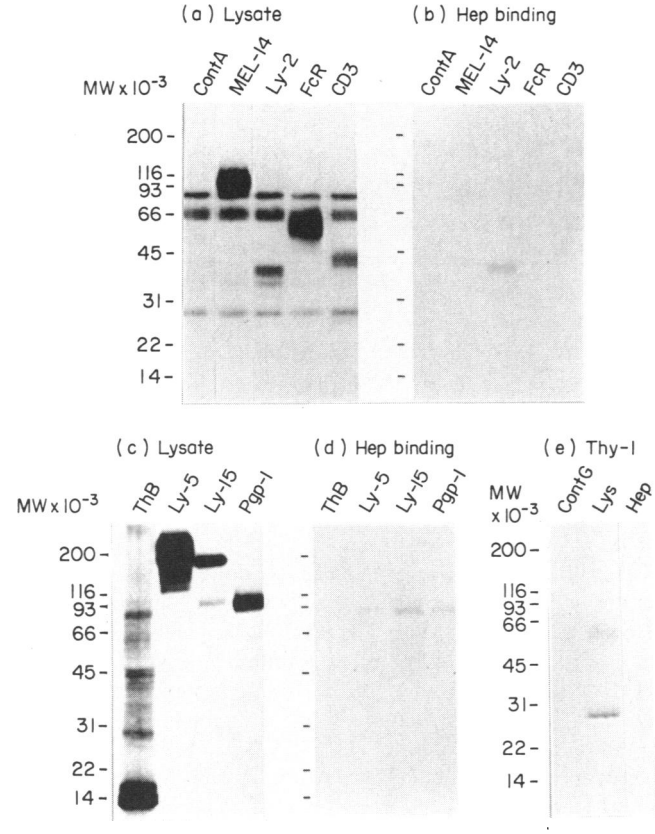

Figure 3. Ability of different splenocyte cell-surface antigens to bind to immobilized heparin. Each mAb was used to monitor the presence of radiolabelled antigen either in an unfractionated splenocyte lysate or in a preparation of ^{125}I-labelled splenocyte molecules eluted from heparin-CMC fibres. Monoclonal antibodies were bound to sheep anti-rat Ig coupled (a and b), protein A coupled (c and d) or protein G coupled (e) Sepharose 4B. In order to detect small amounts of radiolabelled antigen in heparin eluates immunoprecipitates were autoradiographed three to four times longer than immunoprecipitates from unfractionated lysates. Molecular weight markers are indicated.

GAG bound to a unique profile of cell surface molecules (Fig. 2). For example, chrondroitin-4-sulphate bound predominantly to molecules with molecular weights of 10,000–20,000, 33,000 and 90,000, whereas chondroitin-6-sulphate bound additional molecules of 40,000 and 60,000 MW. The reactivity of particular molecules also varied between GAG, for example a 90,000 MW protein on splenocytes reacting strongly with chondroitin-4-sulphate and chondroitin-6-sulphate, moderately with dermatan sulphate and weakly with hyaluronic acid and heparan sulphate. A similar 90,000 MW protein was detected on thymocytes but it reacted strongly with all GAG. Additional differences were seen between the splenocyte and thymocyte receptor profiles, the latter expressing a similar pattern of cell surface molecules which bound to each GAG but with additional molecules of 190,000 and 250,000 MW when compared with spleen. Finally, the molecules which bound to endogenous splenic GAG and heparin included all the major GAG-binding proteins detected on splenocytes and thymocytes. It should be noted that, with the exception of the 90,000 MW protein, a similar range of heparin-binding molecules was detected on purified thymocyte membranes.[17]

Identification of GAG-binding proteins on splenocytes

To determine if any of the cell-surface molecules which bound to GAG corresponded to known lymphocyte cell surface antigens, immunoprecipitation studies were performed on ^{125}I-labelled splenocyte molecules eluted by 2 M NaCl from heparin-coupled CMC fibres. Preparations of heparin-binding molecules were used as they contained all GAG-binding molecules detected (Fig. 2). It was found that mAb against the cell-surface antigens MEL-14, Ly-2, FcR, CD3, ThB, Ly-5, Ly-15, Pgp-1 and Thy-1 immunoprecipitated molecules of the appropriate MW from ^{125}I-labelled splenocyte lysates (Fig. 3a, c, e). In the case of rat mAb the immunoprecipitations were performed with sheep anti-rat Ig beads and, due to residual cross-reactivity of these beads with mouse Ig, radiolabelled surface Ig was present in all immunoprecipitates (Fig. 3a). Subsequent analysis of heparin-binding molecules revealed that, with the exception of the Ly-2 antigen which weakly bound to heparin coupled fibres, neither surface Ig nor any of the cell-surface antigens tested were heparin-binding (and hence GAG-binding) molecules (Fig. 3b, d, e). The faint band seen in the anti-Ly-15 and anti-Pgp-1 immunoprecipitates (Fig. 3d) probably represents non-specific binding of a prominent 90,000 MW heparin-binding protein rather than the Ly-15 and Pgp-1 antigens.

Table 2. Ability of different lymphoma cell lines to enter spleen

Cell lines failing to enter spleen	Cell lines entering spleen
EL-4 (thymoma)	BCL.1 (B cell lymphoma)
BL/VL3 (thymoma)	$RD10_s$ (B cell lymphoma/hybridoma)
MBL-2 (thymoma)	*$R1^+$ (thymoma)
RK4.7 (pre-T cell)	*$R1^-$ (thymoma)
C6VL/1 (thymoma)	CL2-FT2 (thymoma)
LSTRA (thymoma)	

H33342-labelled cells (2×10^7) were injected intravenously into recipient mice and spleens removed 2 hr post-injection. Splenic entry of fluorescent cells was determined by fluorescence microscopy.

*$R1^+$ and $R1^-$ are closely related cell lines which only differ in H-2 antigen expression.[28]

Migration behaviour of lymphoma cell lines

In order to further assess the functional relevance of GAG-binding molecules of lymphocytes, particularly in terms of lymphocyte migration, the next series of experiments examined GAG-binding proteins on lymphoma cell lines which differed in their migration behaviour. However, before such an analysis could be undertaken the migratory properties of several lymphoma cell lines had to be established.

Initially eleven lymphoma cell lines were examined for their ability to enter spleen, each cell line being labelled with the fluorescent dye H33342 and splenic entry of fluorescent cells being quantified 2 hr post-injection. It was found that six of the cell lines failed to enter the spleen whereas five lines gained access (Table 2). Four of the cell lines were chosen for further study, namely BCL.1, $RD10_s$ and $R1^+$, which entered the spleen, and EL-4, a cell line which failed to enter. A more detailed analysis of the migration behaviour of these four cell lines is presented in Table 3. Compared with H33342-labelled splenocytes the splenic entry of $R1^+$, BCL.1 and $RD10_s$ was 9·3%, 33·6% and 62·8% respectively, whereas there was negligible entry by EL-4. Entry of the cell lines into other lymphoid organs, namely PLN, PP and MLN, was found to be unrelated to splenic entry. For example, $R1^+$ and $RD10_s$ did not appreciably enter PLN but both entered spleen. Furthermore, PLN and PP entry did not appear to be related, as shown by the migration of $RD10_s$ to PP but not to PLN. In contrast, MLN entry appeared to occur as a combination of both PLN and PP entry, as would be expected from earlier specificity of migration studies.[21]

Entry of the lymphoma cell lines into the liver and lungs was also quantified to determine if trapping of cells in these organs indirectly affected splenic entry. Although some lines were trapped in the liver and lungs more effectively than normal splenocytes this did not correlate with lack of splenic entry. For example, there was less trapping of the non-splenic homing cell line EL-4 in the liver and lungs than the lines $R1^+$ and BCL.1 which entered the spleen.

Finally, the positioning of H33342-labelled cells was visualized by fluorescence microscopy of whole spleen sections (Fig. 4). Lymphoma cell lines $R1^+$, BCL.1 and $RD10_s$ migrated into the red pulp and marginal zones of the spleen, with few cells entering the white pulp, a localization pattern maintained up to 24 hr post-injection (data not shown). In contrast, migration of fluorescent splenocytes into the white pulp was clearly evident 2 hr post-injection (Fig. 4). EL-4 acted as a control in these experiments, this cell line failing to enter the spleen (Fig. 4), as expected from earlier studies (Table 3).

Identification of GAG-binding molecules on lymphoma cell lines

Following the identification of lymphoma cell lines with different migratory properties, attempts were made to identify GAG-binding molecules on these cells and correlate them with lymphoid organ entry. ^{125}I-labelled cell-surface molecules from different cell lines were bound to a variety of GAG immobilized on CMC fibres and the bound molecules characterized by SDS-PAGE. Each cell line expressed a distinct profile of GAG-binding molecules with a range of molecular masses (Fig. 5); all GAG-reactive proteins detected interacting with heparin (data not shown). $R1^+$ expressed the greatest range of GAG-binding molecules with molecular weights ranging from 10,000–200,000. $RD10_s$ and BCL.1 expressed more restricted profiles of GAG binding molecules, the most prominent protein being a 90,000 MW species with BCL.1 and 58,000–66,000 MW proteins in the case of $RD10_s$. In contrast, EL-4 expressed a small number of low molecular weight (10,000–18,000 MW) GAG-binding molecules.

A detailed comparison of the GAG-binding molecules expressed by the different lymphoma cell lines revealed that no one GAG-binding protein could be correlated with the entry of the cells into a particular lymphoid organ. For example, expression of a 90,000 MW GAG-binding protein was not related to splenic entry as only two ($R1^+$ and BCL.1) out of three of the splenic homing lymphomas expressed the molecule. The presence of this molecule also did not correlate with PLN, PP and MLN entry. A similar situation applied with each GAG-binding molecule of a particular molecular weight.

DISCUSSION

This paper describes attempts to isolate and characterize GAG-binding molecules on the surface of lymphocytes and lymphoma cell lines and relate the expression of these molecules to lymphocyte migration behaviour. Initial binding studies with radiolabelled GAG (Fig. 1) and rosetting studies with GAG-coupled SRBC (Table 1) revealed that there are receptors on lymphocytes for the major classes of GAG. However, the study clearly demonstrated that lymphocytes bind heparin much more avidly than other GAG species. Nevertheless, the binding affinity of murine splenocytes for bovine heparin ($1{\cdot}1 \times 10^{-6}$ M) is approximately 20-fold lower than that calculated for the endogenous heparin-like molecules in murine spleen.[10] Presumably heparin-like GAG in murine spleen have unique structural features which result in more avid recognition by murine splenocytes, a property not unexpected for an endogenous ligand.

Binding of radiolabelled cell-surface molecules to immobilized GAG revealed that lymphocyte populations and lymphoma cell lines differ markedly, in terms of molecular mass, in the profile of GAG-binding molecules they express on their surfaces. For example, of the four lymphoma cell lines examined each gave a characteristic pattern of GAG-binding molecules, some molecules being unique to a particular cell line and others

Table 3. Entry of H33342-labelled lymphoma cell lines into different organs

	% entry relative to splenocytes					
Cell line	Spleen	Peripheral lymph node (PLN)	Peyer's patch (PP)	Mesenteric lymph node (MLN)	Liver	Lungs
EL-4	0·5±0·3	0	0	0	157·9±22·1	66·7±20·9
$R1^+$	9·3±2·0	0	7·9±3·5	1·2±1·2	321·1±34·7	266·7±53·3
BCL.1	33·6±5·8	6·9±0·5	25·6±6·7	54·2±6·2	215·8±30·5	377·8±33·3
$RD10_s$	62·8±10·9	1·2±0·5	174·4±11·8	45·3±3·6	73·7±23·2	55·6±17·6

H33342-labelled cells (2×10^7) were injected into recipient mice and organs removed 2 hr post-injection.

Means and standard errors were calculated from five counts taken from each of three mice (spleen) or one mouse (all other organs).

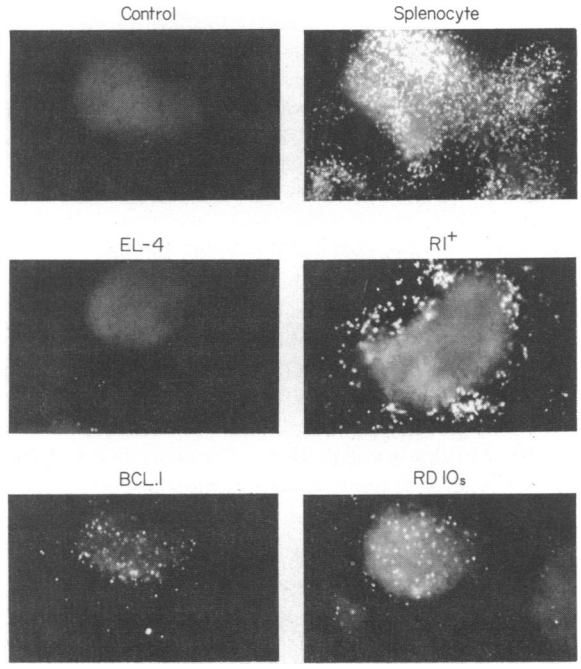

Figure 4. Positioning of H33342-labelled lymphoma cell lines EL-4, $R1^+$, BCL-1 and $RD10_s$ compared with H33342-labelled splenocytes in spleen at 2 hr after intravenous injection of 2×10^7 fluorescently labelled cells into syngeneic recipient mice. Control represents splenic section from an uninjected animal and depicts the autofluorescence of the white pulp of the spleen.

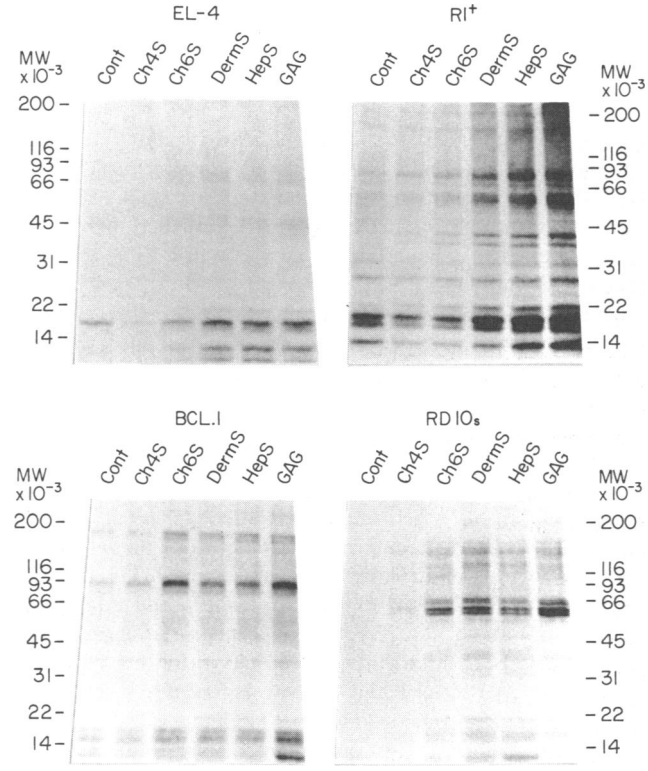

Figure 5. Analysis of ^{125}I-labelled lymphoma cell surface molecules which bind to immobilized GAG. Bound molecules were run on a 8–18% gradient SDS–PAGE gel and gels were autoradiographed for 5 days. Molecular weight markers are indicated. Ch4S, chondroitin-4-sulphate; Ch6S, chondroitin-6-sulphate; DermS, dermatan sulphate; HepS, heparan sulphate; GAG, endogenous splenic GAG.

being shared by some of the lines (Fig. 5). Furthermore, splenocytes differed from thymocytes by possessing a unique family of cell-surface molecules which reacted with each GAG (Fig. 2). This difference may be due to splenocytes being a much more heterogeneous population of cells.

Immunoprecipitation studies with a limited number of mAb were used to try and identify the GAG-binding molecules on splenocytes. It was found that with the exception of Ly-2 (murine CD8), which bound weakly to heparin, none of the GAG-binding molecules corresponded to any of the cell-surface antigens tested and, in particular, molecules associated with cell adhesion such as MEL-14, Ly-15 (CD11/CD18) and Pgp-1 (CD44). Thus, some of the GAG-binding molecules identified in this study may be cell-surface antigens which are not identified by currently available mAb. This is probably the case with the 90,000 MW GAG-binding protein, additional studies indicating that this protein is not an integral membrane protein but is probably bound to the cell surface via an inositol phosphate receptor (M. G. Bradbury and C. R. Parish, manuscript in preparation). However, a much larger collection of mAb needs to be tested to establish the unique nature of the other GAG-binding molecules. In this context it is noteworthy that murine Ly-5 and Thy-1[17] and human CD2[22] and CD4[23,24] have been

shown to be sulphated polysaccharide-binding proteins, although murine Ly-5 is the only one of these molecules with a substantial binding affinity for sulphated GAG such as heparin. Earlier studies in this laboratory clearly established that the Ly-5 (CD45, T200) antigen expressed by murine thymocytes is a heparin-binding molecule,[17] the 190,000 MW GAG-binding molecule detected on thymocytes in this study (Fig. 2) being Ly-5. Thus, it is of particular interest that splenocyte Ly-5 failed to bind to heparin (Fig. 3). Since Ly-5 can potentially exist in eight distinct forms due to differential splicing of the three N-terminal exons of the molecule in a cell-type specific manner,[25] our data would suggest that the thymocyte form of this molecule expresses a heparin-binding site which is lost on the splenocyte form. In fact, it appears that thymocyte Ly-5 lacks the three N-terminal exons of the molecule whereas peripheral lymphocytes express multiply spliced forms of Ly-5.[25]

A major aim of this study was to attempt to correlate the expression of particular GAG-binding molecules on the surface of lymphocytes with the ability of lymphocytes to enter and position themselves within lymphoid organs. By examining four lymphoma cell lines with well-characterized migration properties it was hoped that GAG-binding molecules which controlled migration may be identified. Although each lymphoma cell line displayed a unique profile of GAG-binding proteins a detailed comparison revealed that no one GAG-binding protein could be correlated with the entry of cells into a particular lymphoid organ. Such a comparison assumes, however, that the molecular weight of cell-surface proteins is unchanged between different cell lines, an incorrect assumption for some cell-surface antigens (e.g. Thomas, 1989)[25]. Furthermore, the relative lack of expression of GAG-binding proteins on the surface of EL-4 (Fig. 5), a lymphoma line which fails to enter any lymphoid organs, supports a possible role for GAG recognition in lymphocyte migration. Another important feature of this study is that the lymphoma cell lines were only used to examine the role of GAG-binding proteins in the entry of lymphocytes into lymphoid organs. Previous studies[9] suggested that sulphated GAG recognition plays a particularly important role in lymphocyte positioning in the white pulp of the spleen. In fact, GAG receptors on lymphocytes may not be involved in lymphoid organ entry as recent studies in this laboratory indicate that mannose recognition is involved in splenic entry (S. A. Weston and C. R. Parish, manuscript submitted for publication) and it appears that sulphated polysaccharides inhibit lymph node entry by acting at the endothelial rather than the lymphocyte level.[9] Unfortunately, all three splenic-homing cell lines examined only entered the marginal zones of the spleen and failed to enter the white pulp, precluding an analysis of the role of GAG-binding molecules in white pulp positioning.

Finally, it should be recognized that although this study has concentrated on examining the role of GAG-binding molecules in lymphocyte migration, there are other potential functions for these molecules. For example, GAG-binding molecules may play an important role in the entry of lymphocytes into inflammatory sites, possibly by interacting with GAG exposed on the subendothelial basement membrane[26] or by acting as chemotactic receptors for GAG released by degranulating mast cells. In a more general sense GAG-binding molecules on the cell surface may act as 'scavenger' receptors for extracellular GAG or may aid the uptake of heparin-growth factor complexes by cells.[27]

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