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Biosynthesis of Glycosaminoglycans by Thymic Lymphocytes. Effects of Mitogenic Activation†

Gerald W. Hart

ABSTRACT: The immune response is regulated by cellular interactions involving thymus-derived lymphocytes. Even though evidence from several systems suggests that proteoglycans or their polysaccharide side chains, the glycosaminoglycans, are important mediators (modulators) of cellular interactions, little is known concerning the biosynthesis or possible functions of these macromolecules in lymphocytes. As an initial step in our systematic analyses of the complex arrays of protein saccharides of lymphocytes, the biosynthesis and secretion of glycosaminoglycans by both unstimulated and mitogenically activated lymphocytes have been investigated. Isolated thymic lymphocytes were labeled with D-[6-^{3}H]-glucosamine and $^{35}SO_4^{2-}$, and the amounts of radioactivity in each family of glycosaminoglycan or other types of saccharides were determined. The data indicate the following: (1) Lymphocytes synthesize and secrete substantial amounts of glycosaminoglycans. (2) Activated lymphocytes have greatly accelerated rates of secretion of glycosaminoglycans, which appear to be more highly sulfated than those of nonstimulated cells. (3) Sulfated glycosaminoglycans of lymphocytes consist largely of chondroitin 4-sulfates, with smaller amounts of heparan sulfates. (4) Lymphocyte stimulation results in a rapid and dramatic increase in the relative proportion of both cell-associated and cell-secreted chondroitin 6-sulfates. (5) Lymphocytes synthesize large proportions of an apparently unsulfated, glycosaminoglycan-like glycoconjugate which is resistant to sequential treatments degrading all known types of glycosaminoglycans. Taken together with previous work which indicates that exogenously added glycosaminoglycans are capable of altering lymphocyte functions, these data suggest that lymphocyte-derived glycosaminoglycans themselves may play an important role in modulating the cellular interactions which regulate the immune system.

Thymus-derived lymphocytes are now known to regulate both cellular and humoral immune responses via specific cellular interactions with the other cellular components of the immune network (Katz, 1977). Evidence has suggested that the cell-surface saccharide moieties on lymphocytes might play essential roles in mediating these important regulatory cellular interactions (Gesner & Ginsburg, 1964; Gesner, 1966; Yachin, 1975; McKenzie et al., 1977; Kieda et al., 1978; Decker, 1980; Higgins et al., 1980; Muchmore et al., 1980). In fact, lymphocytes can be quantitatively separated into functional subclasses on the basis of the types of terminal saccharides exposed on their cell surfaces (Reisner et al., 1976a,b; Kimura et al., 1979; Mishell & Shiigi, 1980). Recently, I have directly shown that asparagine-linked oligosaccharides on the surfaces of stimulator splenic lymphocytes are required for the allogeneic stimulation of thymic lymphocytes to occur (Hart, 1982), and Black and co-workers have presented evidence for the involvement of asparagine-linked saccharides in the lymphocyte-mediated, *H-2* restricted cytolysis of virus-infected cells (Black et al., 1981).

Allogeneic, antigen- or lectin-induced blast transformation of lymphocytes produces rapid and profound alterations in the surfaces of these cells such as changes in permeability to cations (Quastel & Kaplan, 1968; Parker, 1974), unmasking or an increase in the number of cell-surface receptors (McCune et al., 1975; Miller et al., 1975), altered membrane fluidity (Yahara & Edelman, 1975), dramatic increase in the cell's net negative surface charge (Sato et al., 1979), increased kinase activity, and extensive alterations in saccharide surface topography, as determined by lectin binding [Speckart et al.

† From the Department of Physiological Chemistry, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205. *Received May 27, 1982.* This work was supported by National Institutes of Health Grant HD 13563 and in part by American Cancer Society Institutional Research Grant No. 1N-11R.

0006-2960/82/0421-6088$01.25/0

(1978), or for review see Wedner & Parker (1976)]. Although several studies have examined the effects of lymphocyte stimulation of glycoprotein synthesis, primarily using sodium dodecyl sulfate gel electrophoresis (Van Eijk et al., 1979; Udey & Parker, 1981), there is comparatively little information with respect to specific saccharide structural alterations which might accompany lymphocyte transformation and the differentiation of lymphocytes to their functional forms (Hayden et al., 1970; Saito et al., 1977; Van Eijk & Mühlradt, 1977).

Glycosaminoglycans (the complex, highly anionic polysaccharide components of proteoglycans) have also been implicated in the regulation of specific cellular interactions, cation or protein fluxes, and growth properties of cells in both developing and adult tissues (Slavkin & Greulich, 1975; Comper & Laurent, 1978; Hay, 1981). Their ubiquitous extracellular location, molecular sieving properties, extremely high charge densities, and enormous potential information content make it likely that they are involved in the modulation of both direct cell–cell interactions and those involving soluble factors. Surprisingly, even though it is now evident that in addition to fibroblasts and chondrocytes, numerous other cell types synthesize complex arrays of these polysaccharides (Margolis & Margolis, 1977; Hart, 1978; Mayne et al., 1978; Hay, 1981), there has been little work which has investigated the possible biosynthesis or functions of glycosaminoglycans synthesized by lymphocytes. However, there have been several studies that investigated the influences of exogenously added glycosaminoglycans on various lymphocyte functions, especially with respect to degenerative autoimmune diseases such as rheumatoid arthritis (Darzynkiewicz & Balaz, 1971; Bey et al., 1979; Nozoe et al., 1979). These studies strongly suggest that glycosaminoglycans are capable of exerting profound and specific influences on immune regulation and function.

The results presented here indicate that relative to classical-type glycoproteins thymic lymphocytes also synthesize substantial amounts of certain classes of glycosaminoglycans. Upon mitogenic activation, the apparent rates of secretion of these polysaccharides rapidly increase by many fold. Furthermore, the glycosaminoglycans that are synthesized after lymphocyte stimulation are qualitatively different than those synthesized by unstimulated cells.

Experimental Procedures

Mice. Highly inbred C57BL/6J mice were obtained from the Jackson Laboratory (Bar Harbor, ME), and breeding colonies were maintained on the premises. Mice utilized in these studies were generally 7–20 weeks of age.

Isolation of Thymic Lymphocytes. Mice were sacrificed by cervical dislocation. Thymi were removed aseptically and transferred to sterile tissue culture dishes (60 mm × 15 mm; Falcon, Oxnard, CA) containing 10 mL of low-sulfate RPMI-1640 media (pH 7.4; formulation 79-5139; Gibco, Grand Island, NY) with 10% (v/v) heat-inactivated (56 °C for 30 min) fetal calf serum, 10 mM *N*-(2-hydroxyethyl)-piperazine-*N'*-2-ethanesulfonic acid (Hepes; Calbiochem, La Jolla, CA), 82.5 μg/mL $MgCl_2 \cdot 6H_2O$, 1.55 μg/mL streptomycin sulfate (Sigma, St. Louis, MO), 100 units/mL penicillin G (Sigma), and 5×10^{-5} M 2-mercaptoethanol (Bio-Rad, Rockville Centre, NY). All dissections and cell preparations were performed at room temperature, since it was observed that chilling on ice substantially reduced the viability of the isolated lymphocytes. Thymi were trimmed free of extraneous tissue, capsular components were removed, and the organs were then transferred to a fresh dish of media. Subsequently, organs were minced into 2–4-mm^2 pieces, and single thymocyte suspensions were obtained by forcing the tissue through a fine mesh stainless steel screen. Small amounts of contaminating fragments were removed by low-speed centrifugation (50*g* for 1 min). Thymic lymphocyte viability was estimated by Trypan blue exclusion and generally ranged between 85% and 95%. Cell number was determined by Coulter counting. Microscopic examination demonstrated that greater than 99% of the cells in the resulting suspension were thymic lymphocytes.

Labeling of Thymic Lymphocytes. All labeling and mitogenic activation experiments were performed at a cell concentration of 2×10^7 cells/mL in the above nutrient media. Unless indicated otherwise (dose–response and time-dependency curves), cells were preincubated, with or without mitogen, for 1–3 h at 37 °C in an atmosphere of 5% CO_2/95% air and a relative humidity of 98%. After preincubation, D-[6-^{3}H]glucosamine (100 μCi/mL, 22 Ci/mmol; Amersham, Arlington Heights, IL) and $H_2{}^{35}SO_4$ (200 μCi/mL carrier free; New England Nuclear, Boston, MA) were added, and incubation was continued for an additional 21–24 h. Except for dose–response experiments, the optimal mitogenic dose of concanavalin A (Sigma) of 2 μg/mL was used. Pokeweed mitogen and phytohemagglutinin were also obtained from Sigma.

Analysis of Glycosaminoglycans. Details of glycosaminoglycan analysis procedures have been described previously (Hart, 1976, 1978). Samples consisting of lymphocytes and/or labeling medium were digested exhaustively with predigested Pronase. Pronase was removed by precipitation with cold 10% (w/v) trichloroacetic acid, and lipids were subsequently removed by chloroform–methanol extraction (2:1 v/v). Aqueous phases were neutralized, lyophilized to dryness, redissolved in 0.1 M ammonium acetate in 20% (v/v) ethanol, and desalted on columns of Bio-Gel P2 (1 × 50 cm; 100–200 mesh; Bio-Rad) equilibrated in the same buffer. Glycosaminoglycans were separated from smaller glycopeptides by chromatography on columns (1 × 200 cm) of Sephadex G-50 fine (Pharmacia, Uppsala, Sweden) equilibrated with 0.1 M ammonium acetate in 20% ethanol. Fractions of 1.2 mL were collected. Aliquots of 0.2–0.4 mL were analyzed for radioactivity by using a Packard 300 CD liquid scintillation spectometer which was calibrated for automatic quench and spillover correction by using external standardization. Liquiscint (National Diagnostics) or Budget-Solve (Research Products) was used as the counting cocktail. Sephadex columns were standardized by elution of blue dextran (M_r 2×10^6, Sigma) and $^{35}SO_4{}^{2-}$.

Heparan Sulfates. Excluded radioactivity from Sephadex G-50 (total glycosaminoglycans) was pooled, lyophilized to dryness, and treated with HNO_2 to selectively degrade N-sulfated glycosaminoglycans, heparins, and heparan sulfates, as described previously (Conrad & Hart, 1975; Hart, 1976). The HNO_2 treatment used here selectively and completely degrades heparins and heparan sulfates after only one treatment (Cifonelli & King, 1972, 1973; Conrad & Hart, 1975). The degradation products of HNO_2 treatment were separated from the remaining resistant glycosaminoglycans (non heparan sulfate glycosaminoglycans) by Sephadex G-50 chromatography as indicated above.

Hyaluronic Acids. Non heparan sulfate glycosaminoglycans that remained excluded from Sephadex G-50 after HNO_2 treatment were pooled, lyophilized, and digested with *Streptomyces* hyaluronidase (Calbiochem). *Streptomyces* hyaluronidase specifically degrades hyaluronic acids, yielding unsaturated tetra- and hexasaccharides as major products (Ohya & Kaneko, 1970). Under the conditions used, a single treatment of non heparan sulfate glycosaminoglycans with *Streptomyces* hyaluronidase resulted in complete degradation

of hyaluronic acid (Hart, 1978; Hart & Lennarz, 1978). Separation of hyaluronidase degradation products from the remaining resistant glycosaminoglycans was again achieved by Sephadex chromatography as described above.

Chondroitin Sulfates. Material which remained excluded from Sephadex G-50 after the above treatments was pooled, lyophilized, and digested with chondroitinase ABC (EC 4.2.2.4; Miles Laboratories, Elkhart, IN) as described previously (Hart, 1976, 1978). Chondroitinase ABC selectively degrades chondroitin, chondroitin 4-sulfate, chondroitin 6-sulfate, dermatan sulfate, and to some extent hyaluronic acid, by a β-elimination reaction which produces characteristic unsaturated disaccharides (Saito et al., 1968; Suzuki et al., 1968). Under conditions used here, chondroitinase ABC was effective in removing all of the chondroitin sulfates from the excluded peak of Sephadex G-50 (Hart, 1976).

Keratan Sulfates. Material remaining excluded from Sephadex G-50 after treatment with Pronase, HNO_2, hyaluronidase, and chondroitinase ABC was pooled, lyophilized, and digested with keratan sulfate endo-β-galactosidase (Keratanase; Miles) as described previously (Hart, 1976; Hart & Lennarz, 1978). Keratanase hydrolyzes the $\beta 1 \rightarrow 4$ galactosidic linkages to *N*-acetylglucosamine which involve nonsulfated galactosyl residues in keratan sulfate like polysaccharides, producing various sized oligosaccharides (Nakazawa & Suzuki, 1975; Hart, 1976).

Glycosaminoglycan Degradation Controls. With each enzymatic or chemical degradation of every set of unknowns, carried out as described above, standard glycosaminoglycans (2 mg) were stimultaneously digested to monitor the efficacy of the reaction. Degradation of these standard glycosaminoglycans was monitored by the cetylpyridinium chloride turbidity assay described earlier (Hart, 1976). In every case, the treatments specifically and completely degraded at least 2 mg of the appropriate glycosaminoglycan standard.

High-Performance Liquid Chromatography of Chondroitinase Digestion Products. Chondroitinase digestion products, which were retarded on Sephadex G-50, were lyophilized and examined for characteristic unsaturated disaccharides (ΔDi-6S and ΔDi-4S)[1] by high-performance liquid chromatography (HPLC) (Lee & Tieckelmann, 1979). Lyophilized samples were dissolved in 0.5 mL of methanol–0.5 M ammonium formate, pH 4.8 (35:65 v/v), and 100 μg of each of the two sulfated and the unsulfated unsaturated disaccharide standards (Miles) was added as unlabeled carrier. Samples were chromatographed over a Spherisorb-NH_2, 5-μm column (4.6 mm × 350 mm; Alltech, Deerfield, IL) with a Micro-Guard, Bio-Sil NH_2 (10 μm; 4.6 mm × 30 mm; Bio-Rad) guard column. Elution of unsaturated disaccharides was performed isocratically with methanol–0.5 M ammonium formate, pH 4.8 (35:65 v/v), at a flow rate of 1 mL/min. Chromatography was performed on a Waters HPLC system consisting of a 6000A pump, M45 pump, Model 660 solvent programmer, Model 450 variable wavelength detector, and Model 730 data module. Elution and recovery of carrier unsaturated disaccharides were monitored by their ultraviolet absorption at 232 nm. Fractions of 300 μL each were collected directly into scintillation vials for determination of radiolabeled chondroitinase ABC digestion products, and vials were counted as described above.

[1] Abbreviations: ΔDi-4S, 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-4-*O*-sulfo-D-galactose; ΔDi-6S, 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-6-*O*-sulfo-D-galactose; HPLC, high-performance liquid chromatography; GAG, glycosaminoglycan.

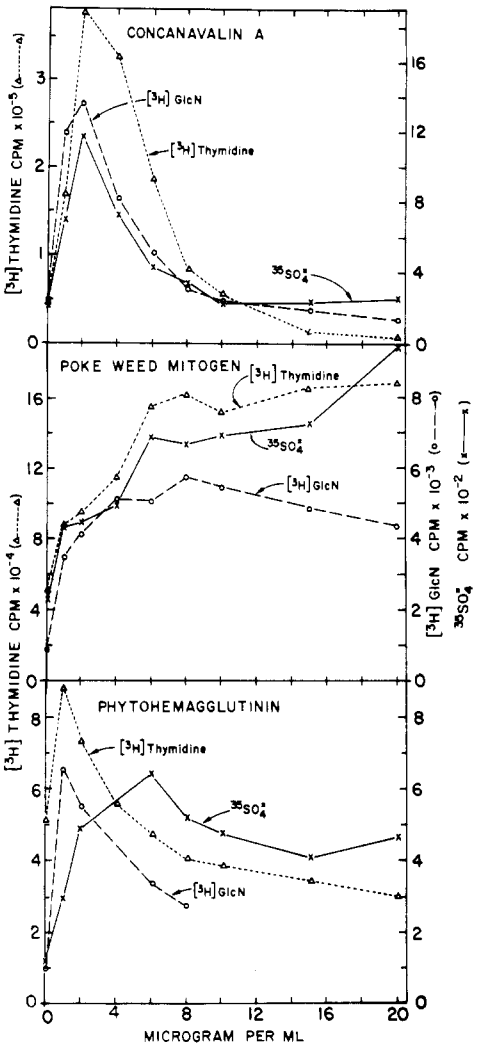

FIGURE 1: Mitogen dose–response curves for the incorporation of $^{35}SO_4^{2-}$, [^{3}H]glucosamine, and [^{3}H]thymidine into trichloroacetic acid insoluble macromolecules of thymic lymphocytes. Lymphocytes were isolated and preincubated for 1 h with or without mitogen, radioactive precursors were then added for 21 h, and cells plus labeling media were subsequently analyzed by trichloroacetic acid precipitation as described under Experimental Procedures. (×) $^{35}SO_4^{2-}$; (○) [^{3}H]-GlcN; (△) [^{3}H]thymidine.

Other Procedures. Trichloroacetic acid precipitation procedures and β-elimination protocols have been described in detail previously (Hart & Lennarz, 1978; Hart, 1982).

Results

Incorporation of Complex Saccharide and DNA Precursors by Thymic Lymphocytes Displays Parallel Mitogen Dose–Response Curves. Preliminary experiments surprisingly indicated that thymic lymphocytes synthesize substantial amounts of glycosaminoglycans. Since it was also observed that the synthesis of these macromolecules was much greater in activated lymphocytes, I investigated the biosynthesis of glycosaminoglycans by both unstimulated and mitogenically activated lymphocytes. Figure 1 shows the mitogen dose–response curves for the incorporation of [^{3}H]thymidine, $^{35}SO_4^{2-}$, and [^{3}H]glucosamine into trichloroacetic acid insoluble macromolecules by thymic lymphocytes. Lymphocytes were stimulated with either of three potent thymic lymphocyte mitogens. Each of these mitogens have very different saccharide binding specifities, concanavalin A (*Canavalia ensiformis* lectin, D-Man or D-Glc), pokeweed [*Phytolacca americana* lectin, *N*-acetyl-D-glucosamine (complex specificity)],

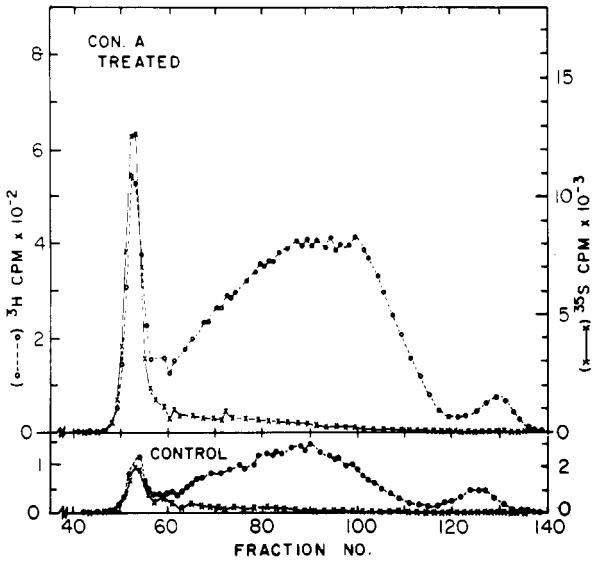

FIGURE 2: Separation of total thymic lymphocyte glycosaminoglycans from lower molecular weight glycopeptides by chromatography on Sephadex G-50. Fractions of 1.2 mL were collected. V_0 equaled approximately 64 mL. Isolated thymic lymphocytes were labeled with $^{35}SO_4^{2-}$ and D-[6-^{3}H]glucosamine in the presence or absence of 2 μg/mL concanavalin A. Cells plus labeling media were digested exhaustively with Pronase and desalted on Bio-Gel P2 prior to chromatography on Sephadex G-50, as described under Experimental Procedures. (×) ^{35}S; (○) ^{3}H.

and phytohemagglutinin (*Phaseolus vulgaris* lectin, complex specificity; Kaifu & Osawa, 1976). As is shown in Figure 1, each T cell mitogen clearly displays a uniquely shaped dose–response curve, and except for $^{35}SO_4^{2-}$ incorporation by phytohemagglutinin-activated cells, the incorporation of both of the complex saccharide precursors parallels that for [^{3}H]-thymidine.

Even though concanavalin A has a very sharp optimal dose response (Figure 1; 2 μg/mL), this lectin was chosen for more detailed studies. When compared to the other lectins at the optimal dose for each, concanavalin A caused a 2.5–4-fold and a 1.5–2-fold greater elevation in [^{3}H]thymidine and saccharide precursor incorportion, respectively.

Glycosaminoglycans Represent a Significant Proportion of the Total Glycoconjugates Synthesized by both Unstimulated and Activated Lymphocytes. Isolated thymic lymphocytes were labeled with D-[6-^{3}H]glucosamine and $^{35}SO_4^{2-}$, in the presence or absence of a mitogenic dose of concanavalin A. Cells and labeling media were left together, digested exhaustively with Pronase, and desalted as described under Experimental Procedures. Figure 2 shows typical results of Sephadex G-50 chromatography of these desalted Pronase digests. Glycosaminoglycans elute in a sharp peak with the void volume. Glycopeptides elute in a broad zone just after the excluded peak. It is apparent from these data that large glycosaminoglycan-like saccharides represent a substantial fraction of the complex saccharides made by both nonstimulated and activated lymphocytes. These data further suggest (as will be discussed below) that mitogenic activation preferentially stimulates incorporation of $^{35}SO_4^{2-}$ and [^{3}H]-glucosamine into this glycosaminoglycan fraction relative to that seen in the lower molecular weight glycopeptides. Also, as has been recently observed in other cell types (Hart, 1978; Lemkin & Farquhar, 1981; Heifetz & Snyder, 1981), lymphocytes synthesize sulfated glycoconjugates coeluting with the glycopeptide fraction. These sulfated glycopeptides are fractionated into several discrete size classes upon rechromatography on high-resolution Bio-Gel P6 columns.[2]

Mitogenic Activation of Thymic Lymphocytes Differentially Stimulates the Incorporation of Radioactivity into Various Types of Glycosaminoglycans. For examination of the types of glycosaminoglycans synthesized by nonstimulated thymic lymphocytes, and also for determination of the effect of mitogenic activation on the biosynthesis of each type of glycosaminoglycan, the total glycosaminoglycan fraction (Figure 2) was analyzed by the sequential-specific degradation procedures described under Experimental Procedures. In parts A and D of Figure 3 are shown typical results of Sephadex G-50 chromatography of the total glycosaminoglycan fractions after they were treated with HNO_2 to specifically degrade the N-sulfated glycosaminoglycans, heparins, and heparan sulfates. Degradation products resulting from HNO_2 treatment of heparan sulfates are well removed from the void volume and are eluted in a characteristic pattern of peaks (Conrad & Hart, 1975). Resistant (non heparan sulfate) glycosaminoglycans continue to elute in the void volume of the column. Clearly, a mitogenic dose of concanavalin A greatly increases the amounts of both $^{35}SO_4^{2-}$ and [^{3}H]glucosamine incorporated into heparan sulfates by lymphocytes (Figure 3A,D). Material remaining excluded from Sephadex G-50 (all of the non heparan sulfate glycosaminoglycans) was pooled, treated with *Streptomyces* hyalurondiase, and again chromatographed on Sephadex G-50. *Streptomyces* hyaluronidase specifically degrades hyaluronates to yield unsaturated oligosaccharides which elute just prior to the inclusion volume on G-50 (Ohya & Kaneko, 1970; Hart, 1978). As is shown in Figure 3B,E, concanavalin A induced activation of lymphocytes has much less relative effect on the incorporation of [^{3}H]glucosamine into hyaluronates than it does on its incorporation into heparan sulfates. Material remaining excluded from Sephadex G-50 after Pronase, HNO_2, and *Streptomyces* hyaluronidase treatments was pooled, treated with chondroitinase ABC, and again chromatographed over Sephadex G-50. Chondroitinase ABC degrades chondroitin sulfates to yield mostly characteristic unsaturated disaccharides which elute near the inclusion volume (Saito et al., 1968). As is shown in Figure 3C,F, chondroitinase ABC completely degrades the remaining glycosaminoglycans which were ^{35}S labeled. Chondroitinase ABC also degrades substantial amounts of the remaining ^{3}H-labeled glycosaminoglycans to products eluting near the inclusion volume of the column in both nonstimulated and activated lymphocytes. Interestingly, most of the ^{3}H radioactivity remains excluded from Sephadex G-50 after all of these treatments (Figure 3C,F). Unlike such material from other tissues (Hart, 1976, 1978; Conrad et al., 1977) this material is also totally resistant to degradation by keratanase,[2] indicating that it is not undersulfated keratan sulfate and also that it is most probably unrelated to recently discovered unsulfated lactosaminoglycans such as erythroglycanin (Jarnefelt et al., 1978; Kukuda et al., 1979; Fukuda et al., 1981). This excluded material is also resistant to exhaustive neuraminidase digestion.[2] Again, mitogenic activation of lymphocytes by concanavalin A stimulates the incorporation of radioactivity into both chondroitin sulfates and into this unidentified-resistant polysaccharide.

Figure 4 and Table I summarize typical results of the glycosaminoglycan analyses of nonstimulated and concanavalin A activated lymphocytes. Glycosaminoglycans represent about 10% of the total ^{3}H radioactivity and above 50% of the total ^{35}S radioactivity incorporated into glycosylated proteins by nonstimulated thymic lymphocytes, whereas the same per-

[2] J. Britz and G. Hart, unpublished results.

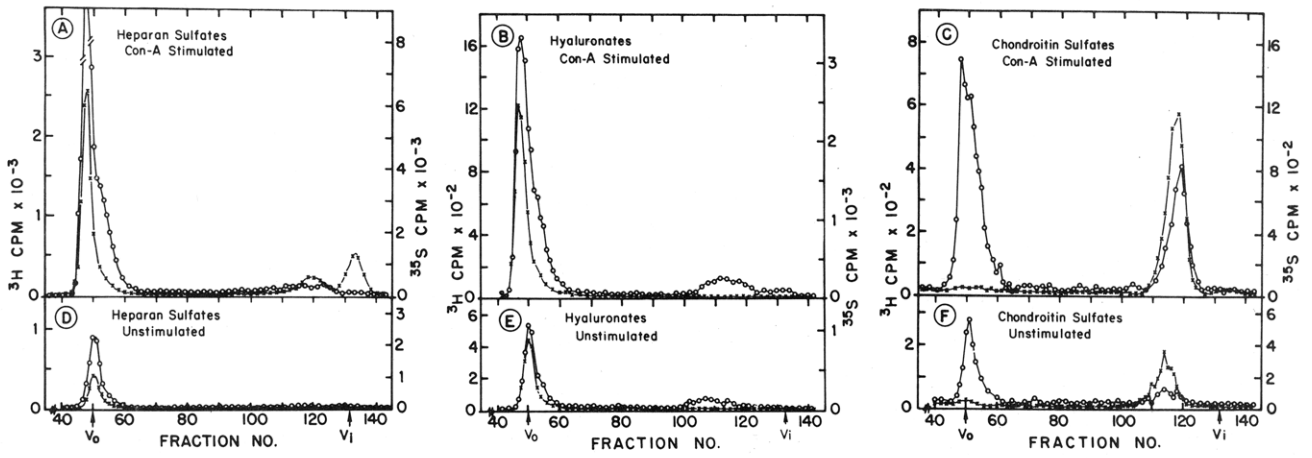

FIGURE 3: Sequential analysis of D-[6-^{3}H]glucosamine- and $^{35}SO_4^{2-}$-labeled glycosaminoglycans of both nonstimulated and activated thymic lymphocytes. Total glycosaminoglycans, which are excluded from Sephadex G-50 (as shown in Figure 2), were pooled and lyophilized. Radioactivity in each family of glycosaminoglycan was determined by specific sequential degradative treatments, in conjunction with Sephadex G-50 chromatography, as described under Experimental Procedures. Fractions of 1.2 mL were collected. (A and D) Separation of non heparan sulfate glycosaminoglycans from degradation products of HNO_2 treatment; (B and E) separation of glycosaminoglycans resistant to digestion with HNO_2 and *Streptomyces* hyaluronidase from degradation products of hyaluronidase treatment; (C and F) separation of glycosaminoglycans resistant to HNO_2, *Streptomyces* hyaluronidase, and chondrotinase ABC from degradation products of chondroitinase ABC treatment. (×) ^{35}S; (○) ^3H.

Table I: Effect of Concanavalin A Activation on the Levels of Radioactivity Incorporated into Various Classes of Glycoconjugates by Thymic Lymphocytes[a]

glycosyl moiety	fold stimulation by Con A	
	^3H	^{35}S
total glycosaminoglycans	5.6	6.5
total glycopeptides	3.5	2.3
heparan sulfates	7.2	15.3
hyaluronic acids	4.1	
chondroitin sulfates	10.4	5.1
unknown polysaccharide(s)	4.6	1.5

[a] Relative to the incorporation of an equal number of unstimulated thymic lymphocytes. For raw data see Figure 4.

centages are about 15% and 74% in mitogenically activated lymphocytes for ^3H and ^{35}S radioactivity, respectively. In nonstimulated lymphocytes, the ^3H radioactivity in each class of glycosaminoglycan is distributed as follows: about 7% heparan sulfates, approximately 15% hyaluronates, about 16% chondroitin sulfates, and the remainder in the unidentified-resistant material. In concanavalin A activated lymphocytes the distribution of ^3H radioactivity into various classes of glycosaminoglycans changes to about 9% heparan sulfates, 11% hyaluronates, 30% chondroitin sulfates, and the remainder in the unknown resistant material. The distribution of ^{35}S radioactivity in sulfated glycosaminoglycans of nonstimulated lymphocytes is about 15% heparan sulfates and 81% chondroitin sulfates, whereas it is about 35% and 64% for heparan sulfates and chondroitin sulfates, respectively, in concanavalin A activated lymphocytes.

Most importantly, the mitogenic activation of thymic lymphocytes dramatically affects the relative levels of radioactivity incorporated into certain specific classes of glycoconjugates, while simultaneously having a much lesser effect on the amounts of radioactivity in others (Table I). [The overall magnitude of the concanavalin A stimulation has been found to be very dependent on the age of the mice; however, the relative effects of different saccharide classes is not age dependent (unpublished results).] In these young adult mice, concanavalin A stimulates the levels of radioactivity (cells and media not analyzed separately; see later) in glycopeptides by only 2–3-fold, whereas that in total glycosaminoglycans, heparan sulfates, hyaluronic acids, and chondroitin sulfates is stimulated 5–6-fold, 7–15-fold, 4-fold, and 5–10-fold, respectively. These data also demonstrate different levels of stimulation of $^{35}SO_4^{2-}$ or [^{3}H]glucosamine incorporation into the same class of glycosaminoglycan. For example, mitogenic activation stimulates $^{35}SO_4^{2-}$ incorporation into heparan sulfates by 15-fold while only stimulating [^{3}H]glucosamine by 7-fold (Table I).

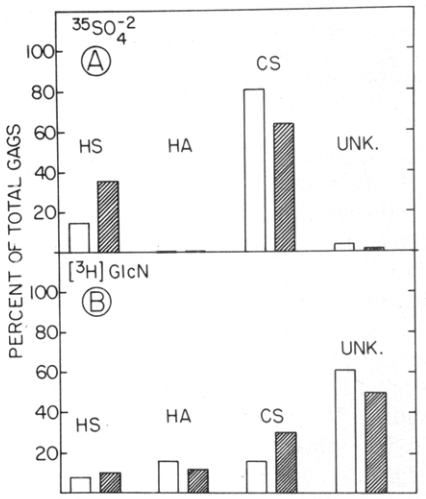

FIGURE 4: Distribution of radioactivity found in each family of glycosaminoglycan synthesized by unstimulated and concanavalin A activated thymic lymphocytes. The distribution of radioactivity was determined as described in Figures 2 and 3 and under Experimental Procedures. HS, heparan sulfates; HA, hyaluronic acids; CS, chondroitin sulfates; UNK, unknown. (A) $^{35}SO_4^{2-}$ data; (B) [6-^{3}H]glucosamine data. Open bar, unstimulated lymphocytes; cross-hatched bar, concanavalin A activated lymphocytes. Total ^3H cpm values in experiments shown were as follows: glycosaminoglycans, 34 000 (control), 195 500 (Con A); glycopeptides, 314 000 (control), 1 110 000 (Con A). Total ^{35}S cpm values in the experiments shown were as follows: glycosaminoglycans, 55 800 (control), 360 000 (Con A); glycopeptides, 56 000 (control), 127 000 (Con A).

Stimulation of Glycosaminoglycan Synthesis and Secretion Occurs Early in the Response of Lymphocytes to Mitogenic Activation. For investigation of the kinetics of the concanavalin A induced effects on both the synthesis and secretion of lymphocyte glycosaminoglycans, thymic lymphocytes were labeled in the presence or absence of the optimal mitogenic

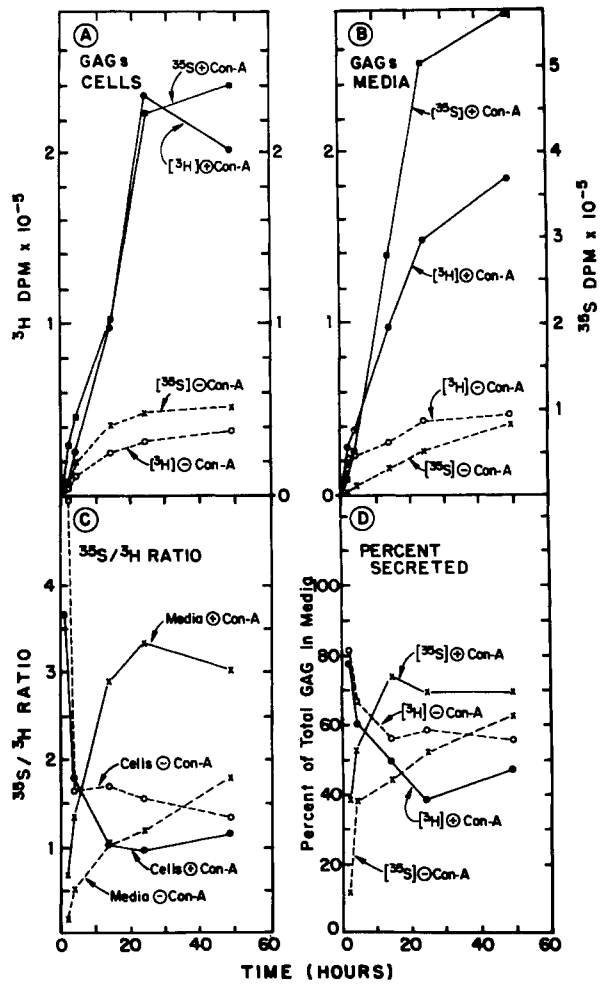

FIGURE 5: Kinetics of incorporation of $^{35}SO_4^{2-}$ and D-[6-^{3}H]-glucosamine into cell-associated and -secreted glycosaminoglycans of nonstimulated and activated lymphocytes. Isolated thymic lymphocytes were radiolabeled either in the presence or in the absence of 2 μg/mL concanavalin A. Labeling was terminated at the times indicated, and the amounts of radioactivity in glycosaminoglycans were determined by Sephadex G-50 chromatography of Pronase digests, as described in Figure 5 and under Experimental Procedures.

dose of lectin, as described earlier, but the amounts of radioactivity incorporated into both cellular and secreted glycosaminoglycans were determined separately as a function of time after addition of mitogen. Figure 5 shows the kinetics of the incorporation of $^{35}SO_4^{2-}$ and [^{3}H]glucosamine into the total glycosaminoglycan fraction of either control or concanavalin A activated lymphocytes. Each point on these curves represents the results of gel filtration analyses as described in Figure 2. Within 2 h concanavalin A causes a significant and reproducible increase in the incorporation of radioactivity into both cell-associated and -secreted glycosaminoglycans, and by 24 h the mitogen activation has tremendously increased the levels of radioactive glycosaminoglycans in both cells and media (Figure 5A,B). Interestingly, by as early as 2 h after concanavalin A addition the $^{35}S/^{3}H$ ratio in secreted glycosaminoglycans increases by over 2-fold relative to nonstimulated cells, and this same ratio increases by over 3-fold by 24 h. By contrast, the $^{35}S/^{3}H$ ratio in cell-associated glycosaminoglycans substantially decreases with time, and concanavalin A actually decreases it even further (Figure 5C). Also, as is shown in Figure 5D, concanavalin A activation of the lymphocytes rapidly results in substantial alterations in the distribution of radioactive glycosaminoglycans between the cells and media. In short, concanavalin A activation of lymphocytes not only

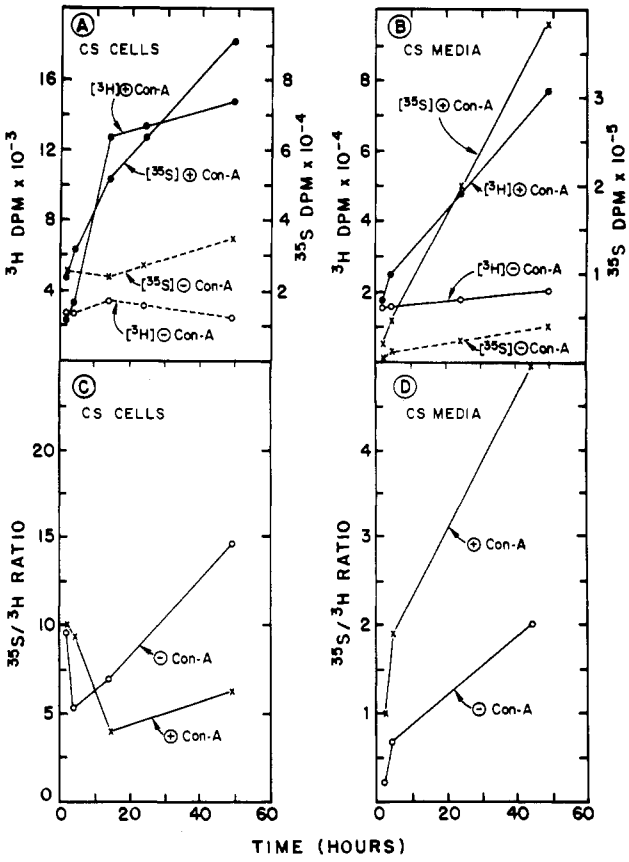

FIGURE 6: Kinetics of incorporation of $^{35}SO_4^{2-}$ and D-[6-^{3}H]-glucosamine into cell-associated and -secreted chondroitin sulfates of nonstimulated and activated lymphocytes. Incorporation of radioactivity into chondroitinase ABC sensitive glycosaminoglycans was determined as described in Figure 3 and under Experimental Procedures. Thymic lymphocytes were activated with 2 μg/mL concanavalin A.

appears to rapidly increase their rates of incorporation of labeled precursors into glycosaminoglycans but also causes them to begin to rapidly secrete glycosaminoglycans which appear to be more highly sulfated than those made prior to stimulation. Results of related studies[3] indicate that many of these mitogen-induced effects can be detected as early as 30 min after addition of mitogen. Figure 6 shows similar kinetic data for the chondroitin sulfates of thymic lymphocytes. Again, mitogenic activation greatly increases the rate of incorporation of radioactivity into, and the secretion of, this particular class of glycosaminoglycan. Strikingly, the $^{35}S/^{3}H$ ratio of secreted chondroitin sulfates is greatly elevated by mitogen, whereas the $^{35}S/^{3}H$ ratio of cell-associated chondroitin sulfates is substantially reduced. Stimulated lymphocytes thus appear to rapidly begin to secrete proportionally large amounts of chondroitin sulfates that appear to be more highly sulfated than those synthesized by unstimulated cells.

Mitogenic Activation of Thymic Lymphocytes Dramatically Increases the Relative Amounts of Chondroitin 6-Sulfates They Synthesize. Chondroitinase ABC digestion products, which were included on Sephadex G-50 (see Figure 3C), were analyzed for the location of the sulfate moiety by HPLC as described under Experimental Procedures. As is shown in Figure 7, even though nearly all of the chondroitin sulfates of unstimulated lymphocytes, in either cells or media, are sulfated at the 4 position of their *N*-acetylgalactosamine residues, mitogenic activation rapidly causes the amounts

[3] L. Powell and G. Hart, unpublished studies.

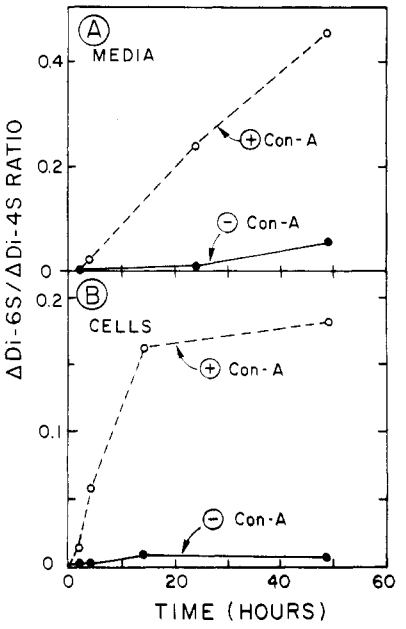

FIGURE 7: Kinetics of mitogen-induced alterations in the sulfation of cell-associated and -secreted chondroitin sulfates of thymic lymphocytes. Chondroitinase ABC digestion products were obtained from Sephadex G-50 chromatography of experiments similar to those shown in Figure 3C,F. Each point on these graphs represents results of HPLC analyses of the characteristic unsaturated disaccharide digestion products of chondroitinase ABC to determine the location of ester sulfate moieties. ΔDi-6S, characteristic disaccharide produced from 6-O-sulfated chondroitin sulfates; ΔDi-4S, characteristic disaccharide produced from 4-O-sulfated chondroitin sulfates.

which are sulfated at their 6 positions to greatly increase. In fact, by 48 h after activation, nearly half of the chondroitin sulfates in the media (Figure 7A) and one-fifth of those in the cells (Figure 7B) are now sulfated at the 6 position of the *N*-acetylgalactosamine residues rather than at the 4 position. Clearly, lymphocyte activation results in dramatic changes in the types of glycosaminoglycans, even within a particular class, which are synthesized and secreted by these cells. These striking changes in glycosaminoglycan metabolism in activated lymphocytes also clearly precede the cell's blastogenic response and immunologically defined differentiation.

Discussion

The aims of these studies were first to determine whether or not lymphocytes synthesize and secrete glycosaminoglycans and second to compare the relative amounts, types, and rates of secretion of these complex polysaccharides in both activated and nonstimulated lymphocytes. Since it was not even known that lymphocytes synthesized significant quantities of proteoglycan-like (glycosaminoglycan-containing) molecules and also because it seemed likely that the lymphocyte proteoglycans would be more complex and very different than those from cartilage, we decided to initially focus on their polysaccharide components, the glycosaminoglycans. [In fact, the size distribution and densities of 4 M guanidine hydrochloride solubilized lymphocyte proteoglycan-like material appear to be very different than those described for other cells, including cartilage or fibroblasts (unpublished observation).] Even though glycosaminoglycan-containing proteins have historically been regarded as connective tissue matrix macromolecules, it is now clear that they are synthesized and secreted by numerous cell types [for review see Hay (1981)], including epithelium, endothelium, retina, nerves, and muscle (Margolis & Margolis, 1977; Hart, 1978; Mayne et al., 1978; Hay, 1981). The present study represents the first systematic investigation of these kinds of complex glycoconjugates in lymphocytes, which are cells generally not considered to establish an "extracellular matrix" in the classic sense of the term.

The results of these investigations indicate the following: (1) Both nonstimulated and stimulated lymphocytes synthesize and secrete substantial amounts of glycosaminoglycans. (2) Sulfated glycosaminoglycans of thymic lymphocytes consist largely of 4-O-sulfated chondroitin sulfates, with smaller amounts of heparan sulfates. (3) Stimulated lymphocytes very rapidly and dramatically increase their rates of glycosaminoglycan secretion relative to nonstimulated cells. (4) Mitogenic activation rapidly increases the relative amounts of 6-O-sulfated chondroitin sulfates which are synthesized and secreted. (5) Lymphocyte activation also results in an increased proportion of heparan sulfates. (6) Over half of the [^{3}H]glucosamine incorporated into the glycosaminoglycan fraction is in a large ($M \geq 10\,000$), unsulfated glycoconjugate which is resistant to sequential treatments capable of degrading all known types of glycosaminoglycans. Such a large proportion of glycosaminoglycan-like material, which is resistant to all of the sequential treatments degrading glycosaminoglycans, has not been observed in previous work with other cell types (Conrad et al., 1977; Hart, 1978; Lemkin & Farquhar, 1981). Related investigations suggest that this novel polysaccharide(s) is (are) made preferentially by the lymphocytes derived from the cortical region of the thymus and is (are) synthesized much less by the more mature medullary lymphocytes.[2]

The stimulation of the incorporation of radioactive precursors into glycoconjugates of mitogen-activated lymphocytes, undoubtedly, in part, reflects the increased metabolism of the cells which are preparing to divide. However, the very rapid increases in secretion of glycosaminoglycans, and the dramatic qualitative changes in saccharides which are synthesized, most certainly reflect the differentiation of the activated thymic lymphocytes to their functional forms. How glycosaminoglycans might be involved in lymphocyte activation or differentiation is unknown, but it is possible that these anionic polysaccharides are involved in mediating the early cation fluxes which are known to play a critical role in lymphocyte blast transformation (Wedner & Parker, 1976; Rasmussen & Goodman, 1977; Hui et al., 1979).

Some of the increases in radioactive saccharide levels of the activated lymphocytes might be due to increased specific activities of precursor pools rather than increased synthesis. However, the intracellular levels of [^{3}H]hexosamines are not substantially different in activated and nonstimulated lymphocytes. In contrast, the levels of uridine-(5′)-diphospho-*N*-acetyl[^{3}H]glucosamine and uridine-(5′)-diphospho-*N*-acetyl-[^{3}H]galactosamine, as determined by HPLC analyses, are rapidly elevated in activated lymphocytes.[3] In any case, the levels of stimulation seen for these nucleotide sugar precursors were similar to those observed for glycopeptides and were substantially less than that noted in the glycosaminoglycans. Furthermore, the observed qualitative changes in saccharides synthesized by activated lymphocytes make it unlikely that the observed effects can solely be explained at the precursor pool level of regulation.

Numerous studies suggest that components of the extracellular milieu such as proteoglycans, glycosaminoglycans, and collagens play a regulatory role in cell and tissue interactions (Slavkin & Greulich, 1975; Lash & Burger, 1977; Hay, 1981). Each stage of morphogenesis and differentiation of tissues is characterized not only by distinct cell types but also by a unique extracellular matrix composition.

Cells contain a wide variety of glycosaminoglycans associated with their surfaces, especially heparan sulfates (Kraemer, 1971; Kraemer & Smith, 1974; Roblin et al., 1975). Interestingly, very recent work indicates that heparan sulfates are able to specifically self-associate and that this property of self-association is altered in heparan sulfates derived from transformed cells (Fransson et al., 1981a,b). Other very detailed studies have convincingly shown that several membrane glycoproteins of baby hamster kidney cells are "hybrids" in that they contain small glycosaminoglycan side chains in addition to their more conventional N- or O-linked oligosaccharides (Baker et al., 1980). Surprisingly, even a previously well-characterized soluble glycoprotein, human thyroglobulin, has recently been found to contain covalently linked glycosaminoglycans (Spiro, 1977). Thus, it is becoming evident that proteoglycan-like (glycosaminoglycan-containing) glycoconjugates of cells such as lymphocytes not only may have unique chemical or physical properties but also may have different overall biological functions compared with those of their counterparts in tissues such as cartilage or cornea.

It has been suggested that cell-surface glycosaminoglycans mediate cell–cell communication (Roblin et al., 1975), shield surface receptors (Kraemer & Smith, 1974), and regulate cell growth (Ohnishi et al., 1975). Sulfated glycosaminoglycans associated with cell surfaces of 3T3 cells are markedly decreased after transformation (Roblin et al., 1975). Addition of exogenous sulfated glycosaminoglycans to cells in culture results in a significant alteration in their mitotic rate (Lippman, 1968). Hyaluronic acids have also been implicated in cellular adhesion that appears to require binding of it to the cell surface (Culp, 1976). Interestingly, hyaluronate produced by cultured cells aggregates lymphoma cells (Pessac & Defendi, 1972; Wateson et al., 1973), while normal lymphocytes and lymphosarcomas are not aggregated by hyaluronic acids.

Exogenously added glycosaminoglycans and/or proteoglycans directly influence the immunological related responses of lymphocytes. Hyaluronic acids derived from rooster comb or human umbilical cord suppress the mitogen-induced stimulation of human peripheral lymphocytes (Darzynkiewicz & Balaz, 1971). The suppressive effects of hyaluronic acids are not due to generalized cellular toxicity and are dependent upon the molecular weight of the hyaluronic acids employed. Hyaluronate also inhibits cytotoxic responses of thymic lymphocytes upon specific target cells and modulates the graft vs. host reactions (Balaz, 1974). Proteoglycans and glycosaminoglycans from normal human cartilage have been shown to modulate both spontaneous DNA synthesis and lectin responsiveness by peripheral blood lymphocytes from normal subjects and from patients with destructive rheumatoid arthritis (Nozoe et al., 1979). Responses of lymphocytes from rheumatoid individuals clearly differed in several respects from those of lymphocytes obtained from normal individuals. Recently, a glycosaminoglycan analogue, dextran sulfate, was shown to replace the requirements for the cellular interaction with thymic lymphocytes usually required to obtain B-lymphocyte proliferation in response to lipopolysaccharide (Wetzel & Kettman, 1981). Thus, the available evidence clearly suggests that proteoglycans and glycosaminoglycans are capable of modulating lymphocyte interactions.

The data reported here, which indicate that lymphocytes synthesize comparatively large amounts of these glycoconjugates themselves, establish the testable hypothesis that lymphocyte-derived glycosaminoglycans may be important in regulating immune function. The murine thymic lymphocyte system, with its highly developed genetics, serology, and quantitative bioassays of specific lymphocyte cellular interactions, provides an ideal model within which to critically examine the possible biological roles of these macromolecules. Studies in progress are investigating the nature of the intact proteoglycans and sulfated glycoproteins of these cells and are attempting to directly ascertain their possible roles in mediating immune cellular recognition and function.

Acknowledgments

I am grateful to Drs. William Lennarz, Judith Britz, and Dan Rossignol for helpful advise and criticism of the manuscript.

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