Dermatan Sulfate Interacts with Dead Cells and Regulates CD5⁺ B-Cell Fate

Implications for a Key Role in Autoimmunity

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CD5⁺ (B-1a) B cells play pivotal roles in autoimmunity through expression of autoreactive B-cell receptors and production of autoantibodies. The mechanism underlying their positive selection and expansion is currently unknown. This study demonstrates that dermatan sulfate (DS) expands the B-1a cell population and augments the specific antibody response to an antigen when it is in complex with DS. DS displays preferential affinity for apoptotic and dead cells, and DS-stimulated cell cultures produce antibodies to various known autoantigens. The companion article further illustrates that autoantigens can be identified by affinity to DS, suggesting that molecules with affinity to DS have a high propensity to become autoantigens. We thus propose that the association of antigens from dead cells with DS is a possible origin of autoantigens and that autoreactive B-1a cells are positively selected and expanded by DS•autoantigen complexes. This mechanism may also explain the clonal expansion of B-1a cells in certain B-cell malignancies. (*Am J Pathol* 2011, 178:2168–2176; DOI: 10.1016/j.ajpath.2011.01.028)

B cells make a major contribution to autoimmunity by secreting autoantibodies and aiding presentation of self-antigens to autoreactive T cells. The therapeutic benefit of B-cell depletion in patients with autoimmune disease underscores their pathogenic role.¹ Of particular interest are B-1a cells, a subclass of B cells with unique developmental origin, surface marker expression, and functional roles.^{2,3} B-1a cells are a distinctive population of CD5⁺ B cells that are enriched for self-reactive B-cell receptors (BCRs) with a restricted repertoire of heavy

and light chains. B-1a cells possess a variety of characteristics that reflect their strong tie to autoimmunity.² B-1a cells produce low-affinity polyreactive and self-reactive antibodies, mainly of the IgM class. These naturally occurring antibodies recognize a variety of autoantigens, such as phosphatidylcholine, DNA, and ribonuclear proteins. They also cross-react with many microbial antigens and thus may provide a natural first line of protection against microorganisms.

Levels of B-1a cells are elevated in various autoimmune diseases, such as systemic lupus erythematosus, rheumatoid arthritis, Sjögren syndrome, and type 1 diabetes mellitus.^{4,5} B-1a cells are also associated with autoimmunity in murine models.⁶ Furthermore, cells of B-1a lineage can undergo malignant transformation to produce B-cell chronic lymphocytic leukemia (B-CLL).³ B-CLL is characterized by the expansion of malignant CD5⁺ B cells, often accompanied by the development of autoimmune symptoms. B-CLL may be an extreme neoplastic example of the wide spectrum of autoimmune disorders due to inaccurate control of specific immune responses.

We demonstrate a pivotal role for dermatan sulfate (DS) in the regulation of B-1a cells. DS, also called chondroitin sulfate B, is a member of the glycosaminoglycan family (formerly also referred to as mucopolysaccharides).⁷ DS is present in many mammalian tissues but is most abundant in skin, blood vessels, heart valves, and tendons. DS can bind various proteins and perform a number of biological functions. We show that DS pro-

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motes B-1a cell expansion by association with dead cells. We propose that DS expands B-1a cells through complexation with autoantigens and that DS•autoantigen complexes selectively stimulate B-1a cells. In a companion article,⁸ we demonstrate that autoantigens in human patients with autoimmune disease share DS affinity as a unifying physicochemical property and can be specifically enriched and identified by affinity to DS. Together, the findings of these 2 studies establish a key role of DS in autoimmunity and autoimmune disease.

Materials and Methods

Synthesis of DS-Cy5, DS-AF568, and DSbt Conjugates

DS (20 mg; Sigma-Aldrich, St. Louis, MO) was mixed with 1 mg of Cy5 hydrazide (GE Healthcare, Piscataway, NJ) or Alexa Fluor 568 hydrazide (Invitrogen, Carlsbad, CA) and dissolved in 1 mL of 0.1 mol/L *N*-morpholinoethane sulfonic acid (MES) buffer (pH 5.0). After adding 20 mg of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC; Sigma-Aldrich), the reaction was performed by mixing on a shaker at 25°C for 4 hours. Another 20 mg of EDC was added, and the solution was left at 4°C for 16 hours. Uncoupled fluorescent dyes and excess EDC were removed by repeated centrifugation with 10-kDa molecular weight cutoff (MWCO) filter membranes until the filtrates were colorless. Purified DS-Cy5 or DS-AF568 was lyophilized and stored at -20°C. For preparation of biotinylated DS (DSbt), 10 mg of DS in 0.2 mL of 0.1 mol/L MES buffer were mixed with 0.5 mg of biotin hydrazide (Pierce, Rockford, IL) predissolved in DMSO, and the reaction was performed at 25°C for 16 hours with the addition of 10 mg each of EDC at the beginning of and 3 hours into the reaction, respectively. DSbt was purified on a PD-10 desalting column (GE Healthcare) and lyophilized.

Preparation of DS-SA and DS-BSA Conjugates

A total of 2 mg of DS in 0.2 mL of PBS (pH 7.2) was mixed with 2 mg of streptavidin (SA; SouthernBiotech, Birmingham, AL) in 0.4 mL of 0.1 mol/L borate-buffered saline (pH 8.2) and 10 mg of EDC. The reaction was performed with gentle shaking at 25°C for 2.5 hours. Another 10 mg of EDC was added, and the reaction mixture was kept at 4°C for 18 hours. The DS-SA conjugate was desalted with a 10-kDa MWCO spin filter and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE). For coating enzyme-linked immunosorbent assay (ELISA) plates, DS-bovine serum albumin (BSA) conjugates were prepared by coupling 12 mg of DS with 12 mg of BSA in 2.4 mL of 0.1 mol/L MES buffer in the presence of 20 mg of EDC at 25°C for 20 minutes. The conjugates were desalted on a PD-10 column and lyophilized.

Cell Culture

Single-cell suspensions were prepared from mouse spleens as described elsewhere.⁹ Unless specified oth-

erwise, spleens from BALB/c mice were used. Spleen cells or enriched B cells were cultured at a density of 1.0 to 1.2×10^6 cells/mL in RPMI 1640 medium supplemented with penicillin, streptomycin, L-glutamine, 2-mercaptoethanol, and 10% fetal bovine serum (FBS) (cRPMI1640-10). Enriched B cells were obtained from spleen cells by depletion of T and other cells using antibodies against CD4, CD43, and Ter-119 (DynaMouse B-Cell Negative Isolation Kit; Invitrogen). DS or modified DS (DS-Cy5, DS-AF568, or DSbt) was used in cell cultures at a concentration of 20 μ g/mL unless otherwise specified. Lipopolysaccharide (LPS) (Sigma-Aldrich) was used at 2 μ g/mL for control experiments. Cell proliferation was measured by incorporation of 5-bromo-2'-deoxyuridine into DNA or uptake of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) by viable cells during the final 16 hours of culture.

Flow Cytometry and Cell Sorting

Cells were typically stained with antibodies or DS derivatives in cRPMI1640-10 medium, 1% BSA in PBS, or 2.5% FBS in PBS at 25°C for 30 minutes, washed twice with 1% BSA in PBS, and subjected to flow analysis. Antibodies used include CD19-biotin, CD19-phycoerythrin (PE)-Cy5, and CD19-PE-Cy7 (clone 1D3; eBioscience, San Diego, CA); CD19-fluorescein isothiocyanate (FITC) and CD19-allophycocyanin (APC) (MB19-1; eBioscience); CD5-PE (53-7.3; eBioscience); CD5-FITC (53-7.3, BD Biosciences); and anti-mouse IgM-PE and IgM-FITC (eB121-15F9; eBioscience). Apoptotic cells were identified by staining with annexin V-FITC (Invitrogen), propidium iodide (PI), and 7-amino-actinomycin D (7-AAD).

Confocal Fluorescence Microscopy

Mouse spleen cells were cultured with 10 μ g/mL of DS-AF568 for 1 to 6 days. Cells were fixed with 1% formalin in PBS at 25°C for 15 minutes and washed twice with PBS. Cells were resuspended in PBS containing 1% BSA or 10% FBS and stained with antibodies or DS derivative at 4°C for 18 hours and then with 50 nmol/L DAPI at 25°C for 15 minutes. In some experiments, nuclei were also stained with SYTOX Green (Invitrogen). Antibodies used include CD19-AF488 (6D5; BioLegend, San Diego, CA), CD19-AF647 (1D3; eBioscience), CD5-FITC (53-7.3; BD Biosciences), and CD5-AF647 (53-7.3; BioLegend). After washing three times with 1% BSA in PBS, cells resuspended in PBS were dropped onto glass slides and mounted with Fluoromount-G (SouthernBiotech). Images were taken on a Zeiss LSM 510 META confocal microscope (Carl Zeiss, Jena, Germany) with a 63 $\times$ Plan-Apochromat objective.

Immunoelectron Microscopy

Murine spleen cells were cultured with 20 μ g/mL of DSbt for 24 hours, washed three times with PBS, fixed with 4% paraformaldehyde at 25°C for 1 hour, and then infiltrated with 2.3 mol/L sucrose in PBS containing 0.15 mol/L glycine at 25°C for 30 minutes. Ultrathin sections were

loaded onto copper grids and blocked with 1% BSA in PBS for 15 minutes. Cells were incubated with polyclonal rabbit anti-biotin for 30 minutes, followed by incubation with protein A-coated gold particles for 20 minutes. Cell sections were then stained with a 9:1 mixture of 2% methyl cellulose and 3% uranyl acetate at 4°C for 10 minutes. Images were acquired on a JEOL 1200 EX electron microscope (JEOL USA, Peabody, MA) at magnifications of 10,000- to 25,000-fold.

Protein Extraction and Western Blotting

Proteins were extracted from mouse spleens or cultured spleen cells with the ReadyPrep cell extraction reagent III (Bio-Rad). After centrifugation at $20,000 \times g$ for 10 minutes at 25°C, the supernatant was collected and dialyzed against 10 mmol/L phosphate buffer (pH 7.2). Proteins were loaded onto 0.5 mL of DS-Sepharose affinity gel[®] and incubated at 25°C for 1 hour. Unbound proteins were washed off three times with 10 mL each of PBS. DS-binding proteins were then eluted with either a two-step (PBS with 0.5 and 1.0 mol/L NaCl) or a four-step (PBS with 0.2, 0.4, 0.6, and 1.0 mol/L NaCl) salt gradient. DS-bound proteins were dialyzed in 3.5-kDa MWCO MINI dialysis units (Pierce), separated on 4% to 12% Bis-Tris gels with NuPage MOPS running buffer (Invitrogen), and stained with Bio-Safe Coomassie Blue (Bio-Rad). Proteins were transferred onto polyvinylidene difluoride membranes and blocked at 4°C overnight with Tris-buffered saline (pH 7.4) containing 2% BSA, 3% casein, and 0.5% Tween 20. CD19 was detected by incubating with polyclonal rabbit anti-mouse CD19 IgG (Santa Cruz Biotech, Santa Cruz, CA) at 25°C for 1 hour, followed by goat anti-rabbit IgG horseradish peroxidase (Santa Cruz Biotech) in blocking buffer at 25°C for 1 hour. Protein bands were visualized with the ECL substrate (Pierce). CD5 was detected similarly with goat anti-mouse CD5 and anti-goat IgG horseradish peroxidase.

Mouse Immunizations

Groups of 8 female BALB/c mice each (Jackson Laboratory, Bar Harbor, ME), 6 to 10 weeks old, were injected intraperitoneally with antigens dissolved in 50 μ L of PBS and emulsified in 50 μ L of aluminum hydroxide gel adjuvant (Sigma). Three doses were administered at 2-week intervals. Blood samples were obtained from mice before immunization and 7 days after each immunization. Antigens tested include (dose per immunization) DS (10 μ g), SA (10 μ g), DS+SA mixture (20 + 10 μ g), DSbt covalent conjugate (10 μ g; dosed by DS content), DS-SA covalent conjugate (10 μ g; dosed by SA content), DSbt•SA non-covalent complex (20 + 10 μ g), and DSbt•SA+DS mixture (20 + 10 + 20 μ g). Before immunization, the optimal mixing ratio of DSbt and SA for complex formation was determined. DSbt and SA were mixed at 1:2, 1:1, 2:1, 3:1, and 4:1 (wt/wt) ratios and incubated at 37°C for 30 minutes. Analysis by 3% to 20% native PAGE showed that all SA formed complexes with DSbt at a ratio of 2:1 (DSbt:SA ratio) (data not shown). To test the adjuvant effect of DS, mouse antibody responses to immunizations with pneu-

mococcal type 14 capsular polysaccharide (Pn14) (10 μ g) and Pn14+DS mixture (10 + 10 μ g) or to a mutant protein of the anthrax toxin protein PA (DNI)^{10,11} (10 μ g) and DNI+DS mixture (10 + 10 μ g) were compared. Pn14 was purchased from the American Tissue Culture Collection and purified as described.¹² DNI was prepared as previously described.^{10,11}

ELISA

Specific and total IgM or IgG (including IgG isotypes) were measured by ELISA in cell culture supernatants or in immunized mouse sera. The following antigens and concentrations were used for plate coating: SA, 10.0 μ g/mL; Pn14, 20.0 μ g/mL; histones (unfractionated from calf thymus; Sigma), 25.0 μ g/mL; double-stranded DNA (dsDNA; activated calf thymus DNA, Sigma), 20.0 μ g/mL; single-stranded DNA (ssDNA), 20.0 μ g/mL; goat F(ab')₂ anti-mouse immunoglobulin, 2.5 μ g/mL; DS-BSA conjugate, 10.0 μ g/mL; DNI, 5.0 μ g/mL; and DS, 20.0 μ g/mL. Negative control wells were coated with 5.0 μ g/mL of BSA. ssDNA was prepared from dsDNA by heating in boiling water for 10 minutes followed by immediate cooling in ice water. Other ELISA steps were performed as previously described.^{11,13}

Results

DS Stimulates CD5⁺ B-Cell Proliferation

We initially screened a series of glycosaminoglycans for their stimulatory effects on primary mouse splenic cells, including DS, heparin, heparan sulfate, chondroitin sulfates A and C, and hyaluronic acid. Cell culture experiments consistently showed that DS was the most potent in stimulating cell proliferation, whereas others had much lower activity. This observation was in agreement with our earlier assessment¹⁴ and later reports.^{15,16} The proliferative effect of DS was dose dependent, and concentrations of 20 μ g/mL or higher significantly stimulated cell proliferation. Moreover, the effect of DS was robustly observed with spleen cells from young or old mice, naive mice, or mice that had been previously immunized with a variety of antigens.

Because primary murine spleen cells include a variety of different cell populations, we analyzed DS-cultured cells by flow cytometry for expression of CD19, CD5, CD72, B220, CD3, CD4, CD8, CD44, CD138, CD44, IgM, IgG, IgD, class II major histocompatibility complex, and others. Among primary murine spleen cells, DS selectively stimulates the expansion of CD5⁺CD19⁺ B cells (ie, B-1a cells) (Figure 1A). This was best seen by fluorescent-activated cell sorting (FACS) after 4 days of culture because longer culturing time typically resulted in cell clusters that were difficult to analyze. By contrast, B cells expanded by LPS for 4 days are predominantly CD5⁻CD19⁺ B cells (ie, B-2 cells) (Figure 1B). We then directly cultured only CD19⁺ B cells purified from total spleen cells with DS, LPS, or medium alone. Again, DS stimulated the proliferation of B-1a cells (see Supplemental Figure S1 at <http://ajp.amjpathol.org>). DS-induced proliferation of B-1a cells was also independently

Figure 1. DS stimulates CD5⁺ B-cell proliferation in cell culture. **A:** Mouse spleen cells were cultured with DS or medium alone (control) for 1 to 4 days. Dot plots of 10,000 total (ungated) events are shown in each graph. Note the progressive expansion of CD5⁺CD19⁺ cells (red circles) in DS but not in medium alone (numbers indicate percentage of events in red circles relative to total events). **B:** DS and LPS stimulate different B-cell populations, as demonstrated by FACS analysis of murine spleen cells cultured with medium alone, DS, or LPS for 4 days. Density plots of 20,000 total events are shown in each graph and each labeled with the percentage of live B cells relative to total cells in the analyzed culture. Note that the CD5 levels of CD5⁺CD19⁺ B-1a cells stimulated by DS are characteristically lower than those of T (CD5⁺CD19⁻) cells. LPS stimulates CD5⁺CD19⁻ B-2 cells. Cells with low CD19 levels (left lower quadrant) are dead B cells. Antibodies: anti-CD5-PE and anti-CD19-PE-Cy5. FSC, forward scatter.

assessed by 5-bromo-2'-deoxyuridine incorporation into DNA and MTT uptake by viable cells (data not shown).

Characteristically, the CD5 expression level of B-1a cells expanded by DS is lower than that of T cells (Figure 1B). These CD5⁺CD19⁺ cells are also positive for IgM, B220, CD72, CD44, CD21/35, and CXCR4. They vary in IgD and CD138 expression, and CD27 expression is low to negative. Moreover, these cells are large, as shown by their increased forward scattering (Figure 1A). The characteristics of these cells suggest that they are activated B cells.

To understand the activity of DS in cell culture, we cultured mouse spleen cells with fluorescently labeled DS (DS-AF568) for various periods and then stained with fluorescently labeled anti-CD5 and anti-CD19. Confocal fluorescence microscopy revealed that DS-AF568 was incorporated into a small percentage (<5%) of cells and, furthermore, that these cells were CD5⁺CD19⁺ B-1a cells (Figure 2A) that were also positive for surface IgM (data not shown). By contrast, cells positive for CD5 only (T cells) or CD19 only (B-2 cells) did not incorporate DS-AF568 (Figure 2A).

Colocalization of DS With CD19 and CD5

To test the involvement of CD19 and CD5 in DS stimulation, we first examined physical colocalization of DS with CD19 and CD5 in cultured cells. Mouse spleen cells were cultured

with DS-AF568 and confocal fluorescence microscopy revealed partial colocalization of DS with CD19 and CD5 both on cell surfaces and inside activated B-1a cells (Figure 2, B and C; see Supplemental Figures S2, A and B, and S3 at <http://ajp.amjpathol.org>).

We then examined *in vitro* the binding affinities of CD19 and CD5 to DS. Mouse spleen cells were cultured with DS for 4 days to obtain large numbers of B-1a cells. Total proteins were extracted from cultured cells, fractionated on DS-Sepharose affinity resin, separated by SDS-PAGE, and interrogated with anti-CD19 and anti-CD5 using Western blot analysis. CD19 was detected in the DS-binding fraction that eluted with 0.4 mol/L NaCl (Figure 2D, left panel). We also extracted proteins directly from naive mouse spleen cells that had not been precultured with DS and found that CD19 displayed similar affinity to DS (see Supplemental Figure S2C at <http://ajp.amjpathol.org>). By contrast, CD5 was present in unbound, weakly bound (eluted with 0.2 mol/L NaCl), and bound (eluted with 0.4 mol/L NaCl) fractions (Figure 2D, right panel).

To further investigate the roles of CD19 and CD5, we cultured CD19-deficient spleen cells from the C.Cg-*Cd19^{tm1(cre)Cgn} lgh^b/J* mouse.¹⁷ Surprisingly, DS stimulated the expansion of CD5⁺ B cells (Figure 2E). These cells were CD19⁻IgM⁺B220⁺, with CD5 expression levels similar to those of normal CD5⁺CD19⁺ B-1a cells. This finding indicates that, although CD19 can physically associate with DS, its expression is not required for DS-induced expansion of CD5⁺ B cells.

Association Between DS and Apoptotic or Dead Cells

To further understand the mode of action of DS in cell culture, we cultured mouse spleen or purified B cells with DS-Cy5 and monitored them by flow analysis. Strikingly, DS-Cy5 was predominantly associated with apoptotic or dead cells (Figure 3A). Apoptotic cells were identified by small size and positive staining with PI or 7-AAD. DS-Cy5 associated with apoptotic cells ranging from early (annexin V⁺) to late (annexin V⁻) apoptotic stages (see Supplemental Figure S4 at <http://ajp.amjpathol.org>). In some experiments, annexin V⁺PI⁺ apoptotic cells were more strongly stained with DS-Cy5 than were annexin V⁻PI⁺ cells, suggesting that DS preferentially associated with apoptotic cells at early stages of apoptosis. Interestingly, staining with DS-Cy5 helped to distinguish two populations of B cells: DS-binding apoptotic or dead B cells with decreased CD19 expression (CD19^{low}DS⁺) and viable B cells with higher CD19 expression but no DS binding (CD19^{hi}DS⁻) (Figure 3B).

To further study the affinity between DS and dead or apoptotic cells, we interrogated different types of lymphoid cells, including primary cells (ie, mouse total spleen or purified B cells) and cell lines such as murine A20 B cells and human WIL2-NS cells. Preferential binding of DS-Cy5 to dead cells was observed in all tested cells (data not shown). Therefore, the affinity of DS to dead cells is not restricted to a single cell type.

Physical association of DS with apoptotic cells, including small apoptotic bodies and fragmented nuclear material,

Figure 2. Involvement of CD19 and CD5 in DS-mediated stimulation of B-1a cells. **A:** Uptake of labeled DS by CD5⁺CD19⁺ cells in mouse spleen cell culture. Only CD5⁺CD19⁺ cells display fluorescence (DS-AF568, false-colored in white). Cells positive for either CD5 (labeled with AF488, red) only or CD19 (labeled with AF488, green) only did not incorporate DS-AF568. Nuclei were stained with DAPI (blue). **B:** Colocalization of DS and CD19 demonstrated by staining DS-AF568–cultured mouse splenocytes with anti-CD19-FITC and DAPI. The example demonstrates partial colocalization (yellow) of DS (red) and CD19 (green) in an activated B cell. **C:** Colocalization of DS and CD5 demonstrated by staining DS-AF568–cultured mouse splenocytes with anti-CD5-FITC and DAPI. DS shows partial colocalization with CD5 (yellow). **D:** Western blot detection of CD19 (**left**) in total unfractionated protein extract and a DS-binding fraction (0.4 mol/L NaCl). Western blot detection of CD5 (**right**) in total unfractionated protein extract and both nonbinding (0.2 mol/L NaCl) and DS-binding (0.4 mol/L NaCl) fractions. Numbers above lanes denote molar concentrations of NaCl used for elution. **E:** DS-mediated expansion of CD5⁺CD19⁺IgM⁺ B cells from CD19-deficient mice (**middle**). Spleen cells from C.Cg-*Cd19*^{tm1(crc)Cgn} *Igh*^b/J mice were cultured with DS for 4 days. Dot plots show 10,000 total events each. Cells in the red circles are negative for CD19 and positive for IgM. Cultures with medium only or LPS (**left** and **right**) are shown as controls. Antibody: anti-CD5-PE. FSC, forward scatter.

was frequently observed by confocal fluorescence microscopy (Figure 3C) and immunoelectron microscopy (Figure 3D; see also Supplemental Figures S1, S6, and S7 at <http://ajp.amjpathol.org>).

Because of the robust association between DS and apoptotic cells, we asked whether DS might reverse the apoptotic process and rescue cells undergoing apoptosis from death, thereby producing the observed expansion of B-1a cells in cell culture. We cultured mouse splenocytes for 24 hours with and without DS and obtained separate fractions of either viable or apoptotic cells by cell sorting. Viable and apoptotic cell fractions were then recultured with DS or medium alone. DS stimulated the proliferation of viable cells only, whereas apoptotic cells were neither rescued nor expanded by DS (see Supplemental Figure S8 at <http://ajp.amjpathol.org>).

DS Stimulates Polyclonal and Antinuclear Immunoglobulin Production in Cell Culture

Because DS leads to expansion of B-1a cells, we asked whether DS also stimulates their differentiation and antibody production. After 7 days, mouse spleen cells cul-

tured with DS produced significantly larger amounts of both IgM and IgG than control cells cultured with medium only (see Supplemental Figure S9 at <http://ajp.amjpathol.org>; additional data not shown). Because B-1a cell expansion was promoted by DS, one would logically ask whether these antibodies are DS specific. Surprisingly, DS-specific IgM and IgG accounted only for small portions of total immunoglobulin in the supernatant, and most antibodies appeared to be directed at other antigens.

We hence hypothesized that DS did not act alone to promote the proliferation and differentiation of B-1a cells but rather by forming complexes with endogenous components from dead cell material. Because of the association of DS with fragmented nuclear material in cell culture and because antinuclear antigen autoantibodies are a hallmark of autoimmunity, we examined whether antinuclear antigen antibodies were produced by DS-cultured cells. Indeed, spleen cells cultured with DS produced significant amounts of IgM specific for several known nuclear autoantigens, including ssDNA, dsDNA, and histones (see Supplemental Figure S9 at <http://ajp.amjpathol.org>).

Noncovalent DS•Antigen Complexes Augment Antigen-Specific Immunoglobulin Responses

To test the hypothesis that noncovalent DS•Antigen complexes promote development of B-1a cells reactive to the antigen, we designed a tunable model antigen system. We chemically attached a small amount of biotin (5%, wt/wt) to DS molecules (DSbt) and used the DSbt•SA complex as a model. Mouse spleen cells were cultured with DSbt•SA complex, DS+SA (noninteracting) mixture, DSbt, DS, SA, or medium only. SA or medium alone did not expand the B-1a cell population. By contrast and similar to DS, DSbt•SA, DS+SA, and DSbt significantly stimulated CD5⁺CD19⁺ B-1a cell expansion (see Supplemental Figure S10 at <http://ajp.amjpathol.org>; additional data not shown). The activity of both DSbt and DSbt•SA suggested that minor modification of DS with biotin did not diminish its potency to expand B-1a cells.

We then assayed for SA-specific IgM and IgG production in *ex vivo* cell culture. When spleen cells from mice that had been immunized with DSbt•SA were cultured, SA-specific IgM was detected in cells cultured with DSbt•SA, whereas DS+SA mixture, DS-SA conjugate, or SA did not stimulate significant anti-SA IgM production (see Supplemental Figure S10B at <http://ajp.amjpathol.org>). Anti-SA IgG was below detectable range in all culture supernatants. The finding that noncovalent complexation of SA with DS significantly boosts anti-SA IgM production suggested that simultaneous signals from both an antigen and DS are required to promote specific B-1a cell responses.

We then tested this hypothesis *in vivo*. Groups of mice were immunized with SA, DS+SA mixture, DS-SA conjugate, or DSbt•SA complex. After three immunizations, both DSbt•SA complex and DS-SA conjugate induced high levels of SA-specific IgG (Figure 4A and Table 1). By contrast, SA alone and DS+SA mixture induced only minute levels of anti-SA IgG.

Although high concentrations of anti-SA IgG were induced in mice immunized with either DS-SA conjugate or DSbt•SA complex, their IgG isotype profiles differed significantly (Figure 4A). DS-SA conjugate induced predominantly IgG1 with barely detectable amounts of IgG3 (IgG1, 83.5%; IgG2a, 10.3%; IgG2b, 4.2%; and IgG3, 2.0%). However, DSbt•SA induced significant levels of both IgG1 and IgG3 (56.6% IgG1, 10.2% IgG2a, 19.4% IgG2b, and 13.8% IgG3). The findings suggest that immunization with DSbt•SA complex induced a strong B-1a cell response because B-1a cells are the main producer of IgG3.⁴

Moreover, when DSbt•SA complex was mixed with free DS (DSbt•SA+DS), the SA-specific IgG response in mice was similarly characterized by significant amounts of IgG1 and IgG3 (61.0% IgG1, 11.5% IgG2a, 13.6% IgG2b, and 13.9% IgG3). However, the total amounts of anti-SA IgG were lower in mice immunized with DSbt•SA+DS compared with DSbt•SA. This finding suggests that the added free DS competitively expanded nonspecific B-1a cells, resulting in an overall smaller fraction of B-1a cells reactive to SA.

Figure 3. Association of DS with apoptotic or dead cells. **A:** Uptake of DS-Cy5 by apoptotic or dead cells. Mouse spleen cells were cultured with DS-Cy5 for 1 day and stained with PI. Gated dead and live cells in the forward/side scatter (FSC/SSC) plot (**left**) are colored in red and green, respectively. Only apoptotic or dead cells in the culture are associated with DS uptake (**right**). Apoptotic (PI⁺) cells appear to associate with slightly more DS than dead (PI⁺⁺) cells. **B:** Binding of apoptotic or dead cells (red) to DS. Cells were cultured with DS for 3 days and stained with anti-CD19-PE and DS-Cy5. Note that all dead cells bind DS-Cy5 irrespective of CD19 expression. **C:** Association of DS with small apoptotic/dead cell bodies. Cultured mouse spleen cells were stained with DS-AF568 (red) and DAPI (blue). **D:** Immuno-gold electron microscopy demonstrating association of DS (small dots) with apoptotic bodies in a fragmented mouse spleen cell. Mouse spleen cells were cultured with DSbt for 1 day and stained with gold-labeled anti-biotin antibodies. The cell shown is fragmented into two condensed nuclear fragments (condensed areas on **lower left** and **lower right**), one granular area, and an autophagosome (**upper right**). Note that DS is mostly associated with the granular material and also with the nuclear membrane. Scale bar = 100 nm.

Figure 4. DS•antigen complexes enhance the IgG and IgM response to antigen exposure. BALB/c mice were immunized with three doses of DSbt•SA complex, DS+SA (noninteracting mixture), DS-SA covalent conjugate, SA, or DS. Serum samples were obtained 7 days after the third immunization. **A:** Mice immunized with DSbt•SA complex or DS-SA conjugate produced high levels of anti-SA IgG. The relative distribution of 4 IgG isotypes is illustrated with colored bars. Note that anti-SA IgG induced by DS-SA conjugate is predominantly IgG1, whereas DSbt•SA complex elicited a significant portion of IgG3. **B:** DSbt•SA complex also enhanced the IgM response to SA. **C:** The IgM response to DS was weak in all test groups (note the smaller scale along the ordinate compared with the other plots). No measurable IgG response to DS was detectable in any group (data not shown). Each graph represents duplicate measurements from eight immunized mice. Bars represent mean values, and upper lines denote plus one SEM.

Repeated immunizations with SA, DS+SA, or DS-SA did not induce any significant anti-SA IgM response. By contrast, DSbt•SA caused a robust increase of anti-SA IgM that was only partially antagonized by competition with free DS (DSbt•SA+DS) (Figure 4B and Table 1).

Surprisingly, and in contrast to the strong anti-SA response, the DSbt•SA complex did not induce a promi-

nent humoral response to DS. Anti-DS IgG was below detection range, and anti-DS IgM was barely detectable and similar for all groups (Figure 4C), consistent with our findings in cell culture.

We then tested whether DS possesses an adjuvant effect on nonbinding antigens. We selected a T-cell-independent type 2 antigen (Pn14) and a T-cell-dependent antigen (the DNI mutant protein of anthrax PA¹¹) as examples. Both Pn14 and Pn14+DS elicited high levels of anti-Pn14 IgM after each dose of immunization but only low levels of anti-Pn14 IgG even after three immunizations. More importantly, neither IgM nor IgG levels differed significantly between mice immunized with Pn14+DS mixture or Pn14 alone (data not shown). Similarly, both DNI+DS and DNI elicited comparably high levels of IgG against DNI (data not shown). These findings indicate that DS does not have any adjuvant effect on immune responses to not physically associated antigens.

Both *in vivo* and *in vitro* antibody responses indicated that complexation of DS with SA significantly influenced the immunoglobulin response. Although SA by itself is a weak T-cell-dependent antigen, SA in the DSbt•SA complex behaves more like a T-cell-independent type 2 antigen, inducing a strong IgM response after each immunization and strong IgG1 and IgG3 responses after booster immunizations. Although the DS-SA conjugate in which DS and SA are covalently and randomly linked demonstrated significantly enhanced anti-SA IgG production, the T-cell-dependent antigenic nature of SA was unchanged. By contrast, the simple mixture of DS and SA did not enhance the anti-SA response. These *in vivo* findings offer further support for the concept that antigen and DS cooperate in an antigen•DS complex to stimulate antigen-specific antibody production.

Discussion

We present evidence to support a key role for DS in the expansion of CD5⁺ B cells. Our studies reveal that non-covalent DS•antigen complexes have the unique capability of expanding B-1a. On the basis of our findings, we propose that DS forms complexes with autoantigens presented by apoptotic or dead cells and that these complexes promote the positive selection and expansion of autoreactive CD5⁺ B cells and the secretion of autoantibodies.

We have previously found that glycosaminoglycans (including DS) may contribute to the pathophysiology of rheumatoid arthritis and autoimmune disease.¹⁴ We then determined that DS potentially stimulates B-cell proliferation (Figure 1; see also Supplemental Figure S1 at <http://ajp.amjpathol.org>) and production of immunoglobulin, particularly IgM, *in vitro*. These findings are in agreement with published results.^{15,16} On further examination, we found that DS specifically promotes the expansion of and antibody production by B-1a cells.

The fact that DS specifically increases CD5⁺CD19⁺ B cells, but not T (CD5⁺) or conventional B (CD5⁻CD19⁺) cells, prompted us to investigate whether CD19 or CD5

Table 1. Time Evolution of Anti-SA Antibody Concentrations in Groups of Mice Immunized With Various Antigens

	Antigen group				
	SA	DS+SA	DSbt•SA	DSbt•SA+DS	DS-SA
Anti-SA IgG, $\mu\text{g/mL}$					
Preimmune	<0.01	<0.01	<0.01	<0.01	<0.01
First bleed	<0.01	<0.01	<0.01	ND	<0.01
Second bleed	3.4 ± 1.3	<0.01	0.8 ± 0.3	ND	28.2 ± 10.0
Third bleed	2.3 ± 1.1	0.01 ± 0.001	72.9 ± 8.9	41.9 ± 3.6	41.8 ± 14.8
Anti-SA IgM, $\mu\text{g/mL}$					
Preimmune	<0.01	<0.01	<0.01	<0.01	<0.01
First bleed	0.2 ± 0.02	0.8 ± 0.1	2.1 ± 0.5	ND	1.0 ± 0.1
Second bleed	0.3 ± 0.03	0.3 ± 0.02	1.6 ± 0.2	ND	0.6 ± 0.06
Third bleed	0.1 ± 0.01	0.1 ± 0.01	3.8 ± 0.7	1.8 ± 0.4	0.2 ± 0.01

ND, not determined.

Values shown denote mean $\pm$ SEM in groups of eight mice each. Blood samples were collected before immunization and 7 days after the first, second, and third immunizations.

directly interact with DS. We initially hypothesized that the BCR coreceptor CD19 might be a receptor for DS based on several observations. First, we found that CD19 has a strong affinity for DS (Figure 2; see Supplemental Figure S2 at <http://ajp.amjpathol.org>). This is in agreement with a previous report that heparan sulfate, a GAG polysaccharide similar to DS, is a ligand for CD19¹⁸ and also weakly mitogenic for B cells. Second, CD19 colocalized with DS. Third, in transgenic mouse models, CD19 has been thought to be required for the development and maintenance of B-1a cells,¹⁹ and CD19 dysregulation has been linked to autoantibody-associated autoimmune disorders, including systemic lupus erythematosus and anti-neutrophil cytoplasmic autoantibodies.²⁰ However, when B cells from CD19-deficient mice¹⁷ were cultured, DS stimulated the expansion of CD5⁺B220⁺CD19⁻ B cells (Figure 2E). This finding argues against a requirement for CD19 in the DS stimulation pathway.

Using fluorescently labeled DS, we found that the most remarkable effect of DS in cell culture was its association with apoptotic or already dead cells (Figure 3; see also Supplemental Figure S4 at <http://ajp.amjpathol.org>). Electron microscopy revealed details of the association of DS with various cellular components, including nuclear fragments, of apoptotic and dead cells (Figure 3; see also Supplemental Figures S5–7 at <http://ajp.amjpathol.org>).

The affinity of DS toward apoptotic cells may modulate their physiologic clearance rate. Furthermore, nuclear and other intracellular autoantigens are known to become redistributed and concentrated within surface blebs on apoptotic cells.²¹ It has been speculated that apoptotic cells may be a source of autoantigens. However, it remains unknown why, from a large total pool of self-molecules, only a relatively small subset of molecules (<1%) become autoantigens and targets of autoimmunity.²² Our study suggests that molecules that can associate with DS possess the propensity to become autoantigens.

We hypothesized that DS exposure leads to a sequence of events as follows. When cells are cultured, cell apoptosis occurs spontaneously. In the presence of DS, DS molecules associate with dying and dead cells in culture. Dead cells and fragmented nuclear material are the source of autoantigens. DS•autoantigen complexes

form and activate B-1a cells by cross-linking autoreactive BCRs on the B-1a cell surface to initiate a cascade of events that culminates in B1-a cell activation and expansion.

To test this hypothesis, we generated a tunable model system using DSbt and SA to form a noncovalent DSbt•SA complex. This was advantageous because, in apoptotic and dead cells, a large number of potential autoantigens would be present simultaneously. As a consequence, DS•dead cell complexes would be difficult to investigate in a controlled manner because one would expect these complexes to produce immunoglobulin to a diverse variety of autoantigens, such as ssDNA, dsDNA, and histones. This is indeed what we found in cell culture studies (see Supplemental Figure S9 at <http://ajp.amjpathol.org>). The antibodies are polyclonal and immunoglobulin specific to any one autoantigen is of rather limited quantity. Hence, the DSbt•SA complex provided a much cleaner model to test the hypothesis.

Antibody responses in immunized mice demonstrated that the DSbt•SA complex profoundly modulated the antigenicity of SA and greatly enhanced the immunoglobulin response to SA. Although SA alone hardly induced any detectable anti-SA immunoglobulin, DSbt•SA induced significant amounts of anti-SA IgM after each immunization and high levels of anti-IgG1 and IgG3 after booster immunizations (Figure 4). The production of IgG3 is a hallmark of the humoral response to T-cell-independent type 2 antigens by B-1a cells.²³ By contrast, the noninteracting mixture of DS+SA enhanced neither IgM nor IgG responses to SA. This observation supports the notion that antigen complexation with DS is required for enhancing the humoral response to a specific antigen.

The binding of self-molecules to DS could change the nature of the self-molecule in such a way that it becomes autoantigenic•DS, a polysaccharide consisting of variably sulfated repetitive structural elements (N-acetyl-D-galactosamine alternating with D-glucuronic acid or more commonly its epimer L-iduronic acid), might simultaneously tether multiple copies of the same or different autoantigens. This would convert isolated autoantigen molecules to polymer-like multivalent antigens. Such multivalent DS•autoantigen complexes might behave analogously to T-cell-independent type 2 polysaccharide an-

tigens²³ and activate B-1a cells by spatially cross-linking multiple low-affinity autoreactive BCRs. The DS moiety of the DS•autoantigen complex might also recruit coreceptors on the same B-1a cells and enhance cell activation, proliferation, and differentiation.

DS is produced during tissue repair or wound healing.⁷ Increased production of DS may be beneficial when dead cells accumulate through high turnover of cells during the injury healing process because the production of natural autoantibodies could facilitate and expedite clearance of dead cells by the immune system. Furthermore, physiologic rates of “background” apoptosis in our body may, together with DS, contribute to sustained renewal of B-1a cells and the naturally occurring antibody repertoire.

As shown in the companion article,⁸ DS has affinity toward a wide variety of human autoantigens. The expansion of B-1a cells by DS may thus be performed by many different molecules. Further investigation of the molecular activities of DS and DS-binding proteins will lead to a more precise understanding of the molecular mechanisms driving B-1a cell activation.

On the basis of this and our accompanying article, we propose that autoantigens are selected because of their affinity toward DS and that DS•autoantigen complexes are responsible for autoreactive B-1a cell development. This theory offers a plausible answer to an ontogenic question central to autoimmunity, namely, “How are autoantigens and autoreactive B-1a cells positively selected?”

Our theory may also offer an explanation for the development of B-CLL. B-CLL is a B-cell malignancy with a number of features shared with autoimmune disorders, particularly the expansion of CD5⁺ B cells and the development of autoimmune manifestation restricted to self-antigens. Although there is abundant evidence that BCR stimulation by autoantigens is involved in the selection and expansion of the malignant clone, similar to the case of B-1a cells in autoimmunity, the nature of the antigens and the mechanisms by which these clones are expanded remain unknown. It is possible that the malignant CD5⁺ B-cell clones are selected and expanded by specific DS•autoantigen complexes. Our theory could offer an approach to the identification of such autoantigens involved in B-cell malignancies such as B-CLL.

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