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Ongoing Immunoglobulin Class Switch DNA Recombination in Lupus B Cells: Analysis of Switch Regulatory Regions

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Abstract

Inflammation and tissue damage in systemic lupus erythematosus (SLE) are mediated by class-switched autoantibodies reactive with nucleic acids, nucleic acid-binding proteins, phospholipids and other self-antigens. While some healthy individuals produce IgM antibodies with specificities similar to those of lupus patients, immunoglobulin class switching to mature downstream isotypes appears to be required for the generation of pathogenic autoantibodies. To characterize the cellular and molecular basis of pathogenic autoantibody production in SLE, we studied the capacity of peripheral blood B cells of naïve phenotype from patients with SLE, rheumatoid arthritis (RA) or healthy control subjects to spontaneously switch to IgG and IgA. In addition, we determined the DNA sequences of the upstream evolutionary conserved sequence (ECS)-I γ promoter regulatory regions that control germline I H -C H transcription and class switch DNA recombination (CSR) to IgG₁, IgG₂ and IgG₄. IgM⁺IgD⁺ B cells from patients with SLE, but not those from RA or healthy control subjects, underwent spontaneous CSR, as assessed by expression of germline I γ 1-C γ 1, I γ 2-C γ 2, I γ 3-C γ 3, I γ 4-C γ 4 and I α 1-C α 1 transcripts, mature (switched) V H DJ H -C γ 1, V H DJ H -C γ 2, V H DJ H -C γ 3 and V H DJ H -C α 1 transcripts and secreted IgG and IgA. Although polymorphic DNA sequences were identified in the ECS-I γ 1, ECS-I γ 2 and ECS-I γ 4 promoter regions, the transcription factor-binding sites that mediate germline I γ -C γ transcription were conserved in patients and controls. However, distinct patterns of nuclear protein binding to an ECS-I γ promoter sequence that contains both positive and negative regulatory elements were observed in SLE patients and controls. These results support a role for exogenous signals, such as through CD40 ligation, rather than altered genomic sequence, in the increased production of class switched autoantibodies in SLE.

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INTRODUCTION

Systemic lupus erythematosus (SLE), the prototype systemic autoimmune disease, is characterized by B cell activation, hypergammaglobulinemia and autoantibodies that mediate tissue injury.^[1–3] The predominant lupus autoantibodies are directed at chromatin and its components, such as histone–DNA complexes, or at peptide or nucleic acid components of complex intracellular particles, such as small nuclear ribonucleoprotein particles and ribosomes.^[2–8] Low affinity IgM antibodies to antigens such as DNA, RNA and Ro are present in sera from normal individuals and asymptomatic relatives of patients.^[9–12] The important attribute of the pathogenic autoantibodies found in patients with SLE, those most prominent in glomerular deposits in lupus nephritis, is that they are IgG produced by an oligoclonal expansion of B cells. These IgG autoantibodies are high affinity, cationic and have undergone somatic mutation.^[1,13–25]

Individual SLE patients and lupus mice indicate that onset of clinical disease is associated with switching of autoantibodies from IgM to IgG.^[26–31] Case reports of selective IgM deficiency, in the setting of IgG and IgA deposits in lupus kidneys, also support the pathogenic importance of class switched antibodies.^[28] Of the four IgG subclasses, IgG₁ and IgG₃ anti-DNA autoantibodies are the most common in patient sera, while IgG₁, IgG₂, and IgG₃, the IgG species with the most potent complement fixing activity, are most frequently found in renal deposits.^[14–16,18–25] The biologic activities conferred by the heavy (H) chain constant (C) segment facilitate transport into extravascular spaces, complement activation and binding to Fc receptors, endowing autoantibodies with the expression of full proinflammatory and pathogenic potential.^[13,18–20,23] The predominance of IgG over IgM among pathogenic autoantibodies and autoantibody-producing B cells in human SLE indicates that IgH chain CSR from IgM to IgG is an important mechanism underlying development of disease.

The generation of pathogenic class-switched autoantibodies in SLE may reflect intrinsic abnormalities in the B cell itself, or may, alternatively, be an expression of augmented cell surface and cytokine-mediated help delivered by T cells, dendritic cells or macrophages. An intrinsic propensity of lupus B cells to proliferate and undergo accelerated class switch DNA recombination (CSR) has been suggested by experiments that documented increased numbers of IgG-secreting cells among high density peripheral blood B cells, even when cultured in the absence of mitogens or exogenous cytokines.^[32,33] Genetic factors may contribute to accelerated Ig class switching, as suggested by the development of IgG anti-histone/DNA autoantibodies and drug-induced lupus in only a subset of procainamide-treated individuals.^[34] In the NZB × NZW F1 murine lupus model, the accelerated Ig class switching observed is controlled by the NZW genome.^[35] In addition to genetic factors that may confer altered sensitivity or regulation of the Ig CSR mechanism in certain individuals, extrinsic signals, particularly those delivered *in vivo* through CD40 and cytokine receptors,

may establish a profile of intracellular signaling molecules that is supportive of Ig CSR.^[36–40]

To further dissect the role of intrinsic genetic factors vs. extrinsic signals to the B cells in the accelerated Ig class switching in SLE, we have determined the expression of intracellular germline I_H-C_H and mature (switched) V_HDJ_H-C_H transcripts and secreted IgG and IgA in SLE and control B cells. In addition, we have analyzed the genomic sequence of the evolutionary conserved sequence (ECS)-I γ promoter regulatory regions in DNA from SLE patients and control subjects. Our data are most consistent with augmented extrinsic help to B cells promoting increased CSR to the pathogenic IgG class.

MATERIALS AND METHODS

Study Subjects

Peripheral blood samples from 19 healthy subjects and 25 SLE patients were used for isolation of genomic DNA. These samples were obtained through the Hospital for Special Surgery SLE Patient Registry and Sample Repository, and the diagnosis was assigned by each patient's physician. Peripheral blood mononuclear cells (PBMC) were also isolated from an additional three patients with SLE, as well as from three rheumatoid arthritis (RA) patients and three healthy controls, and used for study of spontaneous Ig class switching *in vitro*. All patients met ACR criteria for the diagnosis of SLE or RA,^[41,42] and the lupus patients were either in remission or adequately controlled for disease activity with therapy.

Cell Preparation

Surface (s) IgM⁺sIgD⁺B cells were prepared by positive selection using anti-human IgD mAb and the Mini-MACS[®] magnetic bead technology (Miltenyi Biotech, Inc., Auburn, CA). Briefly, PBMC were harvested from freshly heparinized blood specimens by centrifugation on a Ficoll-Hypaque gradient (Sigma Chemical Company, St. Louis, MO), washed three times with PBS and resuspended in endotoxin-free RPMI 1640 medium (Life Technologies[™], Inc., Gaithersburg, MD) supplemented with 20 mM Hepes, L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin and 10% FBS (Life Technologies[™], Inc.). PBMC were depleted of T cells by rosetting with AET-treated sheep red blood cells and then incubated (2×10^7 to 5×10^7) for 30 min at 4°C with 200 μ l of fluorescein (FITC)-conjugated mouse mAb to human IgD in 80 μ l of PBS supplemented with 0.5% BSA. After two washes with PBS, 100 μ l of colloidal superparamagnetic anti-FITC (isomer I) MicroBeads[™] were added. Following this multistep selection, >97% of these resulting IgD⁺ cells were viable as tested by Trypan blue exclusion.

Phenotype Analysis by Flow Cytometry

B cell preparations were assessed for cell surface phenotype by immunofluorescence analysis and flow cytometry. B cells were incubated for 30 min on ice with a mAb and then washed with PBS containing 3% BSA. Mouse FITC- or phycoerythrin (PE)-conjugated mAbs to the following human Ags were used: CD19, CD23 and CD80 (Becton Dickinson Immunocytometry Systems, San Jose, CA), CD71 (Dako Corporation, Carpinteria, CA), IgM and IgD (Sigma Chemicals Company). Flow cytometric analysis showed that >99% of

these cells were CD19⁺, sIgM⁺ and sIgD⁺. As positive control, B cells from the three healthy subjects were stimulated for 48 h with human trimeric CD40L-leucine zipper IgG fusion protein (htCD40L) (Immunex Corporation, Seattle, WA).

Measurement of Ig-producing B Cells

sIgM⁺sIgD⁺ B cells from SLE patients and healthy subjects were distributed at 5×10^4 /well in 96-well microculture plates and cultured for five days. The degree of spontaneous Ig class switching was analyzed by measuring the levels of (i) germline I γ 1-C γ 1, I γ 2-C γ 2, I γ 3-C γ 3 and I γ 4-C γ 4 transcripts or (ii) mature V_HDJ_H-C γ 1, -C γ 2, -C γ 3 and -C γ 4 transcripts using the methods previously reported.^[43] RNA from unstimulated B cells (3×10^6) was isolated using RNeasyTM Total RNA Kit (Qiagen Incorporation, Valencia, CA) and then reverse transcribed, in equal amounts, using the M-MLV reverse transcriptase (SuperScriptTM Preamplification System for first strand cDNA synthesis, Life TechnologiesTM Incorporation) in conjunction with a poly(dT)12–18 primer. For the amplification of germline I_H-C_H transcripts, PCR was performed in 50 μ l volume using the GeneAmp[®] PCR Reagent Kit (Perkin Elmer Cetus Corporation, Norwalk, CT). cDNAs obtained from 2, 1, 0.5 and 0.25 μ g of RNA were used as templates in B cells from healthy control subjects, SLE patients or RA patients. A forward primer end-labeled with [γ -³²P]ATP and recognizing each I_H region was paired with a reverse primer recognizing the related C_H region. The forward and reverse primers used for the amplification of the sterile germline I_H-C_H and mature (switched) V_HDJ_H-C_H transcripts, as well as of the control β -actin transcripts, were as previously described.^[43,44] The PCRs consisted of 25 cycles, each entailing 1 min of denaturation at 94°C, 1 min annealing at 68°C and 1 min extension at 72°C. The amplified cDNAs were fractionated on an agarose gel to isolate the cDNA band of appropriate molecular size for measurement of radioactivity and comparison of curves constructed using the different amounts of cDNA template from different experimental samples.

To measure secreted Ig, B cell cultures were established as above and culture supernatants harvested at day eight. Secreted IgM, IgG and IgA were measured by ELISA as previously described.^[43,44]

Characterization of Genomic DNA Sequence of IgH Chain ECS-I γ Promoter Regulatory Regions

Genomic DNA was extracted from PBMC using the Blood and Cell Culture DNA Midi Kit (Qiagen Incorporation) according to the manufacturer's instruction, and fragments encompassing the ECS-I γ 1, ECS-I γ 2 and ECS-I γ 4 promoter regions and corresponding transcription initiation sites were isolated by PCR amplification using subclass-specific primers as indicated in Table I. The PCR was performed in a volume of 25 μ l using 100–200 ng genomic DNA and PCR Master Mix (Promega, Madison, WI). PCR amplification cycles consisted of 5 min denaturation at 96°C, followed by 28 cycles (for γ ₁ and γ ₄) or 30 cycles (for γ ₂). The conditions were: 96°C for 30 s, 56°C (for γ ₂ and γ ₄) for 20 s, 55°C (for γ ₁), and 72°C for 1 min. The last cycle was followed by 10 min at 72°C to complete the extension. The PCR products were subject to electrophoresis on a 2% agarose gel. The band was carefully picked up and recovered using the QIAquick Gel Extraction Kit (Qiagen

Incorporation). Sequencing reactions were performed using 100 ng of PCR product. All sequences that were not clearly demonstrated by direct sequencing were confirmed by ligation of the purified PCR product into the pGEM-T vector (Promega), followed by transformation into DH5 competent cells. The recombinant plasmid was prepared and sequenced using 500 ng plasmid DNA by the dideoxy chain termination method.

Extraction of Nuclear Proteins

Nuclear extracts were prepared from PBMC using a modification of the procedure by Schreiber *et al.*^[45–47] Briefly, PBMC were washed in cold PBS and 10^6 cells resuspended in 400 μ l buffer A (10 mM HEPES, 10 mM KCl, 0.1 M EDTA, 0.1 mM EGTA, 1 mM DTT, 1 mM Na_3V , 0.5 mM PMSF, 10 μ l Leupeptin + Antipain + Aprotinin); 25 μ l 10% NP-40 were added and the mixture placed on ice for 15 min, followed by centrifugation for 15 s. The supernatant was completely discarded and the pellet resuspended in 50 μ l cold buffer C (20 mM HEPES, 0.4 M NaCl, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 10 μ l Leupeptin + Antipain + Aprotinin), incubated on ice for 30 min, vortexing vigorously every 5 min, and centrifuged at 4°C for 10 min. The extracts were aliquoted and stored at –70°C.

Electrophoretic Mobility Shift Assay (EMSA)

The probe used in the EMSA was a 30 bp oligonucleotide (forward: 5'-CTGGGGGCCTGAGCTGTGATTTCCTAGGAA-3'; reverse: 5'-TTCCTAGGAAATCACAGCTCAGCCCCCAG-3') (Invitrogen Company, Carlsbad, CA). The forward and reverse oligonucleotides were labeled separately using [γ - ^{32}P]ATP. The reaction, including 2 μ l of oligonucleotide (1:75 pmol/ μ l), 1 μ l T4 polynucleotide kinase 10 $\times$ buffer, 1 μ l [γ - ^{32}P]ATP (3000 ci/mmol at 10 mci/ml), 5 μ l nuclease-free water and 1 μ l T4 polynucleotide kinase (5–10 u/ μ l) for a total volume of 10 μ l, was incubated at 37°C for 10 min. Before annealing the labeled oligonucleotides, they were purified using QIAquick Nucleotide Removal Kit (Qiagen Incorporation).

The binding assay was performed in a binding buffer [20% glycerol, 5 mM MgCl_2 , 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, 50 mM *Tris*-HCl (pH 7.5), 0.25 mg/ml poly(dI-dC)]. Assay samples of 10 μ l included 1 μ l labeled probe and 5 μ g of nuclear protein. In competition experiments, competitor fragments were added before the probe in 100-fold excess. For supershift experiments, 2 μ l of anti-Ku (p70/p80) antibody (Ab-3, Neo Markers, Fremont, CA) (1:0 mg/ml) were incubated with nuclear extracts for 30 min before addition of probe. The complete reaction mixture was incubated for 10 min at room temperature before loading on a 8% nondenaturing polyacrylamide gel in 0.5 $\times$ TBE buffer; bands were then visualized by autoradiography.

RESULTS

Isolation of IgM⁺IgD⁺ B Cells of Naïve Phenotype from Lupus and Control Donors

To test the hypothesis that SLE B cells have an increased propensity to undergo class switching to downstream Ig isotypes, particularly IgG, “naïve” B cells that are not in an activated or differentiating state must be studied. By definition, naïve B cells are

lymphocytes that have not yet encountered antigen and have not, therefore, undergone any degree of differentiation. Naïve B cells are sIgM⁺sIgD⁺ and do not express the costimulatory molecules, such as CD80 and CD86, that are induced by engagement of CD40 by CD154 (CD40L) and are crucial in B cell/T cell interaction. In addition, naïve B cells express no or minimal levels of “activation” and/or “proliferation” markers, such as CD23 and CD71. sIgM⁺sIgD⁺ B cells that meet these requirements were isolated from the peripheral blood of SLE patients either in remission or adequately controlled for disease activity with therapy and compared to sIgM⁺sIgD⁺ B cells purified from the peripheral blood of three healthy subjects and three RA patients (Fig. 1). While the intensity of fluorescence of CD19 (pan-B cell antigen) was variable among individual donors, whether lupus patients or controls, virtually all cells expressed that B cell marker along with sIgM and sIgD, typically expressed by naïve B cells. In contrast, CD80 and CD86, markers of activation through the CD40 pathway, were negative or nearly negative on all cells.

In agreement with previous reports documenting the presence of variable proportions of sIgM⁺sIgD⁺ naïve B cells expressing CD23, some 10% of healthy control B cells and 25% of SLE B cells expressed this activation marker.^[48] While CD23 expression may indicate *in vivo* exposure to activating stimuli, including CD40L, the level of CD23 expression was modest when compared to healthy control B cells stimulated *in vitro* with CD40L-293 cells (Fig. 1, second group of histograms). The transferrin receptor (CD71) was present on 3% of normal B cells and on 9% of SLE B cells. In summary, both normal and SLE sIgM⁺sIgD⁺ B cells displayed an immunophenotypic pattern that is consistent with that expressed by naïve B cells, although the possibility remains that some cells had experienced CD40 ligation, or exposure to other stimuli, such as B lymphocyte stimulator (BLyS), at some point *in vivo*. The B cell phenotypes demonstrated are representative of the B cell populations utilized in all functional assays of the Ig switching potential of SLE and healthy control B cells.

Lupus sIgM⁺sIgD⁺ B Cells Switch Spontaneously and Effectively to Downstream Isotypes

CSR is always preceded by germline I_H-C_H transcription of the upstream and downstream C_H genes that will be involved in the recombination event, as well as other downstream C_H genes.^[49] Transcription starts from the “initiation of transcription” I_H region and proceeds through the switch (S) and C_H region sequences to generate I_H-C_H transcripts. To determine whether naïve lupus B cells undergo spontaneous CSR, sIgM⁺sIgD⁺ B cells from SLE patients were purified from freshly isolated circulating PBMC by positive selection for human IgD, cultured in the absence of any activating stimuli and compared in the same experiments to sIgM⁺sIgD⁺ B cells from healthy subjects for the presence of germline I_H-C_H and mature variable-diversity-joining (VDJ)-C_H transcripts. Germline I_γ1-C_γ1, I_γ2-C_γ2, I_γ3-C_γ3, I_γ4-C_γ4 and I_α1-C_α1 transcripts were expressed by SLE B cells but not B cells from healthy controls (Fig. 2). Likewise, mature V_HDJ_H-C_γ1, V_HDJ_H-C_γ2, V_HDJ_H-C_γ3 and V_HDJ_H-C_α1 transcripts were detected in SLE but not control B cells. To determine whether the SLE B cells secreted Ig of class switched isotype, the supernatants from unstimulated cultures were harvested after eight days and assayed for the concentration of IgM, IgG and IgA by specific ELISAs (Fig. 3). B cells from the SLE patients produced as much as 500 ng/ml of IgG that included all subclasses but was particularly abundant in IgG₁ and IgG₃ (not shown). In addition, SLE sIgM⁺sIgD⁺ B cells responded with higher efficiency to a

switch-inducing stimulus with production of IgG protein. SLE sIgM⁺sIgD⁺ B cells produced higher levels of IgG when cultured with low numbers of CD40L-293 cells and IL-4 than did sIgM⁺sIgD⁺ B cells from a healthy subject (Fig. 4). Taken together, these data extend previous observations using unfractionated high density SLE B cells that showed spontaneous production of downstream Ig isotypes.^[32]

Analysis of the DNA Sequence of ECS-I γ Promoter Regulatory Regions

The high rate of switching to IgG in lupus B cells may be due, among other factors, to an altered structure, and therefore function, of the promoter regulatory ECS-I γ sequences that lie upstream of the human S γ regions and include sequence motifs that form the structural bases of transcriptional regulation by CSR-inducing signals (Fig. 5).^[46,47,49–52] To investigate whether genomic variability among individuals in the sequence of these essential promoter regulatory elements might account for spontaneous CSR and high IgG production in SLE B cells, the ECS-I γ segment of the γ 1 region, producing a predominant subclass of pathogenic lupus antibodies, along with those generating IgG₂ and IgG₄, were sequenced from SLE and healthy control individuals. Reliable data from ECS-I γ 3 was not consistently obtained due to its high level of sequence similarity to the ECS-I γ of other subclasses and therefore are not presented here.

Sequence data obtained from 25 SLE patients and 19 healthy donors largely confirmed the previously published sequences for the upstream promoter regulatory regions of γ 1, γ 2 and γ 4, with minor differences noted in the γ 2 and γ 4 sequences (Fig. 5).^[53,54] Bases distinct from previously published sequences were found in γ 2 at position 518 (inserted G) and in γ 4 at positions 207 (inserted C), 335 and 337 (inserted C and G) and 395, 396, and 398 (inserted Gs). These changes are either conserved with other γ sequences or are outside of the ECS-I γ region, enriched in transcription factor-binding sites important for both positive and negative control of IgG germline transcription.^[46,47]

Two polymorphic sites in the ECS-I γ 1 promoter regulatory region were identified in sequences from the SLE and healthy control samples, although neither of those sites is located within the ECS region (Fig. 5 and Table II). Moreover, the alleles at the polymorphic sites are similarly distributed among SLE and control donors, suggesting that the polymorphisms are unlikely to contribute to variable expression of S transcripts. In addition to these sites, a single mutation (or a very rare polymorphic allelic variant) in one SLE sample was observed at position 210 of ECS-I γ 1, a site that is not within a predicted transcription factor-binding site.

Sequence data were also obtained for ECS-I γ 2 and ECS-I γ 4 in a small group of patient and control samples. Two polymorphisms were documented at positions 437 and 456 of ECS-I γ 2, both 3' of the ECS and neither within a predicted transcription factor-binding site. The distribution of the alleles at this site is equivalent between SLE patients and controls. One mutation was noted at position 297 in ECS-I γ 4 in an SLE sample. Once again, this mutation is not located in a putative binding site for a regulatory protein. In summary of the sequence data, several polymorphic sites were noted in the ECS-I γ 1 and ECS-I γ 2 promoter regulatory regions, but these were not located in segments that would be predicted to be important for

transcriptional regulation and were not differentially distributed between SLE patients and controls.^[46,47]

Protein Complexes Binding to Oligonucleotides Containing the Switch Repressor Element in SLE and Control B Cells

A recent report from one of our laboratories (P.C.) has defined a S regulatory element (SRE) in the 3' portion of ECS-I γ 3 that mediates repression of germline I γ 3-C γ 3 transcription and inhibition of subsequent CSR.^[52] Mutational analysis of this SRE has identified an ATTT motif, imbricated with the GAS motif that binds STAT6, as a site that is occupied by HoxC4, Oct-1 and Ku70/Ku86 in resting sIgM⁺sIgD⁺ B cells. Dissociation of this protein complex from the SRE upon engagement of B cell CD40 is associated with generation of germline I γ 3-C γ 3 transcripts and CSR to IgG3.^[46,47]

To begin to characterize the role of factors extrinsic to S genomic DNA in the augmented class switching observed in SLE, we performed EMSAs using nuclear extracts from PBMC from eight SLE and four healthy control subjects. Nuclear proteins bound to a ³²P-labeled double stranded oligonucleotide corresponding to position 343 to 371 of ECS-I γ 3, used in previous published studies, were resolved and detected on a polyacrylamide gel (Fig. 6A). A high mobility DNA–protein complex consisting of several component bands was detected in all nuclear extracts, although the intensity of those bands was, in general, greater in the SLE samples than in the healthy control samples. In addition, most samples demonstrated a band with slower and more variable mobility. This band was also more prominent in the SLE samples than in the healthy control samples. Competition with an unlabeled probe ablated both protein complexes in SLE and control samples (Fig. 6B). As Ku70 had previously been demonstrated to be an important component of the repressor complex bound to the ATTT motif in ECS-I γ 3,^[52] the capacity of anti-Ku70 antibody to shift or diminish either of the demonstrated DNA–protein complexes was investigated. While anti-Ku antibody inclusion in the reaction mixture resulted in ablation of the higher mobility band in samples from three healthy controls, that band was only partially decreased in three of four SLE samples (Fig. 6C). The slower mobility complex was not consistently altered by the anti-Ku70 antibody and remained more prominent in the SLE samples. The requirement in these experiments for sufficient nuclear protein material did not permit the analysis of protein complexes in isolated sIgM⁺sIgD⁺ B cells. Nonetheless, these data suggest relatively less binding of the Ku70 repressor complex to the ECS in SLE compared with healthy control cells and are consistent with previous CD40-mediated B cell activation. Additional experiments will be required to identify the slower mobility complex, although STAT6, binding to a motif that overlaps the repressor-binding motif, is a possible candidate.

DISCUSSION

In these studies we have demonstrated spontaneous Ig CSR of isolated B cells from patients with SLE. The B cells were selected based on expression of both sIgM and sIgD, characteristic of cells that have not yet received differentiation signals from T cells, typically through CD154–CD40 interaction, or by the actions of cytokines, such as BlyS, a TNF family member with the capacity to promote CSR.^[36–40] The phenotype of the selected B

cells was consistent with that of naïve or resting B cells, although we cannot rule out prior exposure to activating stimuli that were either below the threshold for induction of cell surface activation molecules or too distant from the time of assay for detection of an activated B cell phenotype. While the SLE B cells did not express high levels of CD23, CD71, CD80 or CD86, several samples did have higher levels of those markers than did control cells. Spontaneous class switching of naïve SLE B cells was documented by demonstration of germline I_H-C_H and mature V_HDJ_H-C_H transcripts for γ 1, γ 2, γ 3, γ 4 and α 1, and by spontaneous production of IgG and IgA protein. S transcripts were not detected in cultures containing naïve B cells from healthy control subjects, and only trace amounts of IgG and IgA were secreted by those cells.

Studies of both human SLE and murine lupus provide strong support for the conclusion that dysfunction or dysregulation at the B cell level contributes to accelerated Ig class switching and pathogenic autoantibody production.^[3,32,33,55–65] Among the many *in vitro* studies documenting B cell hyperactivity in SLE are data that directly address the relative expression of IgM and more mature Ig isotypes. Analysis of polyclonal Ig secretion by lupus B cells has shown that spontaneous IgA and IgG synthesis is increased 20-fold over normal, while IgM is increased only two-fold.^[58] Quantification of spontaneous IgM and IgG anti-DNA autoantibody producing lupus B cells *in vitro* demonstrated high levels of IgG anti-DNA, but barely detectable IgM anti-DNA autoantibody.^[65] Rubin *et al.* have used the syndrome of procainamide-induced lupus to identify, over time, the antibody correlates of clinical lupus-like disease.^[34] While a variety of antinuclear antibodies were found in the vast majority of patients who received procainamide treatment for more than one year, only those who showed a rapid switch to production of IgG antibodies reactive with a histone–DNA complex developed disease. In some of these patients, there was no apparent lag between the detection of IgM and appearance of IgG autoantibodies.^[34]

In the NZB/W F1 murine lupus model, the earlier onset of disease in females than males correlates with the earlier switch to IgG antibodies, and only those mice that produce IgG autoantibodies develop severe nephritis. *In vitro* studies of lymphocytes from NZB/W F1 mice have shown increased numbers of colony forming B cells and spontaneous IgM and IgG secreting B cells.^[66] These abnormalities are independent of T cells and can be transferred to nonautoimmune, syngeneic recipient mice by bone marrow progenitor stem cells. Moreover, the threshold signals required by NZB/W B cells for activation and proliferation are lower than are those for normal B cells.^[67] While these and other data support the important role of altered B cell function and particularly Ig CSR in pathogenic autoantibody production, it has been difficult to define factors that are intrinsic to the B cell in either human or mouse lupus. In fact, studies in which alpha–beta T cell receptor-positive cells are eliminated support an important role for those cells in pathogenic autoantibody production.^[68]

To consider contributions to augmented Ig class switching that clearly originate in the B cell, we studied the ECS-I_H sequences that promote and regulate CSR. A body of evidence suggests a crucial role for the sequence that lies upstream of each S region in initiating and directing germline transcription of the unrearranged downstream C_H gene and in the induction of CSR.^[46–49,69,70] Polymorphisms within the human Ig S μ region have been

defined by RFLP analysis. Of particular relevance for our studies, polymorphisms in the human $S\mu$ region have been described, one of which is associated with renal disease.^[71–76] Another IgH chain gene polymorphism has been negatively associated with SLE in a defined ethnic group.^[77] We are not aware of other analyses of ECS-I γ genomic DNA in a patient population.

Given the observation of spontaneous Ig CSR in isolated sIgM⁺sIgD⁺ SLE B cells, we addressed the hypothesis that intrinsic variability in the promoter of Ig S regions might confer increased production of S transcripts and promote switching from IgM to production of downstream Ig isotypes. Several polymorphic variant DNA sequences were identified within the ECS-I γ regions among the SLE and control DNA samples characterized. However, none of those polymorphisms segregated with lupus disease. While not ruling out a contribution of genomic variability in other segments of Ig genes to the observed augmented IgG production in SLE, our data have analyzed the promoter regulatory regions most directly implicated in control of CSR and have not identified a genomic basis for increased switching. The data, then, direct attention to factors either extrinsic to the B cell or between cell surface and nucleus in order to account for the altered regulation of Ig production observed.

Like most other events in the antigen-dependent phase of B cell differentiation, T helper (Th) cells play a crucial role in allowing B cell activation and terminal differentiation through signals generated in the context of Th cell/B cell contacts.^[36–39,78] Following activation by antigen, Th cells express CD154 and release several cytokines, including IL-2, IL-4, IL-10 and TGF- β , that can drive class switching.^[77] Soluble mediators derived from dendritic cells, such as BLyS (BAFF), also promote B cell differentiation and Ig class switching.^[40] CD154, IL-6, IL-10 and BLyS are all reported to be increased in SLE and could mediate the observed spontaneous Ig CSR.^[79–83]

To begin to address the contribution of extrinsic factors to spontaneous Ig CSR in SLE B cells, we pursued an analysis of the protein complexes bound to a recently described repressor motif in the ECS-I γ promoter regulatory regions.^[47] This motif binds a complex containing HoxC4, Oct-1 and Ku70/Ku86 and overlaps a motif that binds STAT6. Association of the complex with DNA in the basal resting state inhibits production of S transcripts. In contrast, dissociation of the complex from the ECS-I γ promoter regulatory region results in the expression of germline I γ -C γ transcripts. Although we were limited to studying unfractionated PBMC due to requirements for large protein quantities, our data, using EMSA, suggest that the nuclear proteins that form complexes bound to the DNA ECS-I γ SRE are distinct in SLE and healthy control cells. While one of the complexes in the control nuclear extracts was diminished by anti-Ku70 antibody, protein complexes from several SLE samples produced bands that were not abrogated by anti-Ku70. Additional experiments are required to characterize the proteins that are differentially present in SLE compared to control B cells. STAT6, a transcription factor induced by IL-4, is a candidate for further study.

The production of class switched, predominantly IgG antibodies with specificity for self-antigens is a hallmark of SLE and contributes to disease pathogenesis through activation of

complement pathways as well as mononuclear phagocyte and neutrophil inflammatory responses. IgM autoantibodies are often present in healthy individuals and, in general, are not pathogenic. The data presented in this study demonstrate the spontaneous production of IgG by SLE B cells of naïve phenotype, but suggest that those cells are likely to have experienced prior activation stimuli *in vivo*. Although our data do not rule out a contribution of genomic variability to increased production of IgG in SLE, they have not implicated polymorphisms in the ECS-I γ promoter regulatory regions. In contrast, an initial analysis of the protein complexes bound to an ECS-I γ 3 DNA containing a repressor motif and a STAT6-binding site suggests that the signals experienced by SLE cells differ from those of B cells from healthy subjects. Taken together, the data are most consistent with a significant contribution of extrinsic signals, possibly those derived from T helper cells that engage CD40, to the spontaneous Ig class switching of SLE B cells. Nevertheless, the contribution of polymorphism in other IgH regulatory regions, such as the IgH chain 3' hs1,2, hs3 and hs4 enhancers,^[84] to the increased CSR observed here in lupus B cells should be addressed.

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Abbreviations

BLyS	B lymphocyte stimulator
C	constant region
CSR	class switch DNA recombination
D	diversity region
ECS	evolutionarily conserved sequence
EMSA	electrophoretic mobility shift assay
H	heavy chain
I	intervening/initiation of transcription region
Ig	immunoglobulin
J	joining region
PBMC	peripheral blood mononuclear cells
RA	rheumatoid arthritis
S	switch region
SLE	systemic lupus erythematosus
SRE	S regulatory element
Th	T helper cell
V	variable region

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FIGURE 1.

Fluorescence flow cytometry analysis of sIgM⁺sIgD⁺ B cells isolated from the peripheral blood of three healthy subjects, three SLE patients, and three RA patients. B cells isolated from each individual, as well as B cells cultured for 48 h with human trimeric CD40L-leucine zipper IgG fusion protein, were analyzed for cell surface markers by flow cytometry. For each marker, solid histograms represent results obtained using the mAb under study, whereas dotted histograms represent data using an isotype matched control mAb with irrelevant binding activity.

FIGURE 2.

Lupus sIgM⁺sIgD⁺ B cells undergo spontaneous CSR to S γ 1, S γ 2, S γ 3, S γ 4 and S α 1. PCR analysis of germline I γ 1-C γ 1, I γ 2-C γ 2, I γ 3-C γ 3, I γ 4-C γ 4, I α 1-C α 1, I α 2-C α 2, I ϵ -C ϵ and productive Ig V_HDJ_H-C γ 1, V_HDJ_H-C γ 2, V_HDJ_H-C γ 3, V_HDJ_H-C γ 4, V_HDJ_H-C α 1, V_HDJ_H-C α 2 and V_HDJ_H-C ϵ transcripts was performed in freshly isolated SLE and healthy control sIgM⁺sIgD⁺ B cells after four days of culture in medium alone. Total RNA was extracted and reverse transcribed into cDNA. The cDNA was amplified by PCR using a forward primer recognizing each of the I_H regions (for germline I_H-C_H transcript amplification) or a consensus sequence within the FR3 region of the V_HDJ_H segment (for mature V_HDJ_H-C_H transcript amplification) and a reverse primer recognizing C γ 1, C γ 2, C γ 3, C γ 4, C α 1, C α 2 and C ϵ C_H1 regions, as previously reported (43). Amplified bands show the expected sizes: β -actin 580 bp, I γ -C γ 1 603 bp, I γ -C γ 2 579 bp, I γ -C γ 3 670 bp, I γ -C γ 4 411 bp and I α -C α 1 1194 bp for germline transcripts; and FR3-C γ 1 420 bp, FR3-C γ 2 420 bp, FR3-C γ 3 420 bp, FR3-C γ 4 420 bp, FR3-C α 1 904 bp, FR3-C α 2 891 bp and FR3-C ϵ 179 bp for productive transcripts.

FIGURE 3.

sIgM⁺sIgD⁺ B cells from lupus patients but not normal subjects or RA patients spontaneously switch to downstream isotypes. sIgM⁺sIgD⁺ B cells from three SLE patients, three healthy subjects, and three RA patients were purified from freshly isolated PBMC, cultured in the absence of any activating stimuli and assessed for their ability to switch to IgG and IgA. After eight days, the culture supernatants were harvested and assayed for IgM, IgG and IgA concentration by specific ELISAs. Depicted are the concentrations of IgM, IgG and IgA measured in two healthy subjects (Control subjects Exp. # 1 and # 2) and one RA patient (Control subject Exp. # 3), and all three SLE patients. The IgG and IgA concentrations in the remaining one healthy subject and two RA patients were negligible and similar to the concentrations of the related subjects depicted here.

FIGURE 4.

Lupus sIgM⁺sIgD⁺ B cells (filled squares) respond with higher efficiency to CSR-inducing stimuli than sIgM⁺sIgD⁺ B cells from a healthy subject (open circles). sIgM⁺IgD⁺ B cells from a SLE patient (#2) and a healthy subject (#2) were cultured with human CD40L-293 cells at different ratios, in the presence of human rIL-4 at 100 U/ml. After eight days, the concentration of the culture fluid IgG was measured and plotted as a function of the CD40L-293/B cell ratio.

1 Y1 AAGCTTCAGA TGAGAGGCCA GGTATTGAGA GGCTGAGATC ACGGGCAGAA TGGTCATAAT
Y2 AAGCTTCAGG TGAGAGGCCCA GGTATTGAGA GGCTGAGACC ACGGGCAGAA TGGTCATAAT
Y4 AAGCTTCAGG TGAGAGGCCCA GGTATTGAGA GGCTGAGACC ATGGGCAGAA TGGTCATAAT

61 Y1 CGCTGCCAGT ATCAGTCCAG CCCCAGGGAC TCAGAGACAG AGAAAAGAGC AGCACACAAG
Y2 CACTGCCAGT ATCAGTCCAG CCCCAGGGAC TCAGAGACAG AGAAAAGAGC AGTGAACAAG
Y4 CGCTGCCAGT CTCAGTCCAG CCCCAGGGAC TCAGAGACAG AGAAAAGAGC AGCACACAAG

121 Y1 GTCTGGG.CTC CCCACCTTCT CCCGTGAGTA CGGGGGAGTA TGGGGGCAGC CACCACCCCC
T/C G/A
Y2 GTCCGGG.CTC CC.ACCTTCT CCAC.GAGTA TGGGGG.....CAGC CACCACCCCC
Y4 GTCCGGGTCTC CCCACCTTCT CCCGTGAGTA GGGGGAGTA.TGGGGGCAGC CACCACCCCC
KB3

181 Y1 ATCCCCACAC ACCCATGAGG CAGCCTCGGC TGTGTCTGGA CTCCCCCTCG CCCTCTGACC
C/T
Y2 ATCCCCACAC ACCCATGAGG CAGCCTCGGC TGTGTCTGGA CTCCCCCTTA CCCTGTGACA
Y4 ATCCCCACAC ACCCATGAGG CAGCCTCGGC TCTGTGTGGA CTCCCCCTCG CCCTCTGACA
KB4

241 Y1 CAGAAACCAC CAGAAGAAAA GGGAACCTCA GGAAGTAAGT GGTGCCGCCG GTTTCATACC
Y2 CAGAAACCAC CAGAAGAAAA GGGAACCTCA GGAAGTAAGC GGTGCCGCCG GTTTCATACC
C/T
Y4 CAGAAACCAC CAGAAGAAAA GGGAACCTCA GGAAGTAAGG GGTGCCGCTG GTTTCATACC
BSAP A

301 Y1 TGTTCCTAGT CTTTGCAGCG TGGAGTTCAC ACACCTGGGG AC[CTGGGGGC CGAGCTGTGA
Y2 TGTTCCTAGT CTTTGCAGCG TGGAGTTCAC ACCCCTGGGG AC[CTGAGGGC CGAGCTGTGA
G/T G/A
Y4 TGTTCCTAGT CTTTGCAGCG TGGAGTTCAC ACCCCTGGGG AC[CTGGGACC TGAGCTGTGA
GAS

361 Y1 TTTCTAGGA A] GACAAATAG CGGCTGACGG CAGGGGCGGG GCTGCCCACA TGTACCTCGC
Y2 TTTCTAGGA A] GACAAATAG CAGCTGACGG CGTGGGCAAG TCTGCCCACA TGTACCGCGC
C/T
Y4 TTTCTAGGA A] GACAAATAG CGGCTGACGG CAGGGGCGGG GCCGCCACA TGTACCTCGC

421 Y1 CAGAACAGGA AGGGCTGAGA CCCCCACCTC GGTGAGTGGG GTCAGCACAG GGCAGGGGCA
Y2 CAAAACAGGA AGGGCTGAGA CCCCCACCTC GGTGAGTAGG GTCAGCACAG GGCAAGGGCA
A/G G/C G/A
Y4 CAGAACAGGA AGGGTTGAGA CCCCCACCTC GGTGAGTGGG GTCAGCACAG GGCAGGGGCA

481 Y1 CAGGCTCGGG AGGAGACAGAGCCTGGGGCG AGCCGTGGGC GCTCCTGGAC CTGAGATGCT
Y2 CAGGCTCGGG AGGAGAAGGA CAGAGCCTGGGTGC AGCCGTGGGC GCTCCTGGAC CTCAGCTGCT
Y4 CAGGCTCGGG AGGAGAAGGA CAG..CCTGGGCGC AGCCGTC.GC GCTCCAG.AC CTGAGCTGCT

FIGURE 5.

Polymorphisms in the ECS- γ 1, ECS- γ 2 and ECS- γ 4 promoter regulatory sequences. The ECS-I γ 1, ECS-I γ 2 and ECS-I γ 4 promoter regulatory region sequences are depicted with the initial base numbered in accord with the publication by Mills *et al.*^[53] The ECS is highlighted in gray and important transcription factor binding sites are noted. A S regulatory element that confers basal negative regulation is bracketed. SLE and healthy control DNA samples were amplified by PCR with primers described in Table I. Forward and reverse primer sequences are boxed. The location of the reverse primer for ECS-I γ 2 is 3' of the

sequence presented in the figure. Sequence data for SLE and control DNA samples were compared to published sequences with GenBank accession numbers AL122127 ($\gamma 1$), U39934 ($\gamma 2$) and HSG481A ($\gamma 4$). Our data for ECS-I $\gamma 2$ and ECS-I $\gamma 4$ showed minor differences from the published sequences^[53,54] (G inserted at position 518 in I $\gamma 2$; C inserted at position 207, C and G inserted at positions 335 and 337, respectively, and G's inserted at positions 395, 396, and 398 in ECS-I $\gamma 4$). These differences are underlined. Two polymorphic sites in ECS-I $\gamma 1$ (positions 124 and 163) are underlined and indicated in bold. A single mutation at position 210 of ECS-I $\gamma 1$, present in one SLE sample, is underlined. Polymorphic sites at positions 437 and 456 of ECS-I $\gamma 2$ are underlined and indicated in bold. Single mutations at positions 280, 320, 352, 407 and 425 were detected, all in healthy control samples, and are underlined. The ECS-I $\gamma 2$ polymorphisms and mutations were confirmed by sub-cloning into plasmid DNA followed by sequencing. A single mutation was detected at position 287 of ECS-I $\gamma 4$ in one SLE patient and is underlined.

FIGURE 6.

Electromobility shift assay analysis of nuclear protein complexes from SLE and healthy controls associated with an ECS-I γ 3 DNA oligonucleotide containing a repressor-binding motif. (A) Nuclear protein extracts of PBMC from four healthy controls and eight SLE patients were reacted with a 32 P-double stranded oligonucleotide (ECS-I γ 3) that included the motif known to bind a complex containing HoxC4, Oct-1 and Ku70/Ku86 as well as a GAS site known to bind STAT6. DNA-protein complexes were resolved on an 8% polyacrylamide gel. (B) Cold unlabeled ECS-I γ 3 probe was included in reactions containing nuclear extracts from three healthy control subjects and three SLE patients and 32 P-labeled ECS-I γ 1 probe. (C) Anti-Ku70 antibody was included in reactions containing nuclear extracts from three healthy subjects and four SLE patients.

TABLE IPCR primers used in analysis of ECS-I γ sequences

Target DNA	Primer sequence
ECS-I γ 1	
Forward	5'-CCAGGTATTGAGAGGCTGAGAT-3'
Reverse	5'-TCTTGGAGCCTGTTTCAGCAT-3'
ECS-I γ 2	
Forward	5'-TCCCACCTTCTCCACGAGTA-3'
Reverse	5'-GGCTCTGCATCCCGTCAT-3'
ECS-I γ 4	
Forward	5'-GGTCTCCCCACCTTCTCC-3'
Reverse	5' CCCAGGCTGTCCTTCTCC-3'

TABLE II

ECS-I γ DNA polymorphisms

Subjects	Base position	N	Genotype	Genotype frequency (%)
ECS-I γ 1				
Healthy Controls	124	19	T/T	100
	163	15	G/G	78.9
		3	G/A	15.8
		1	A/A	5.3
SLE	124	23	T/T	92
		2	T/C	8
	163	21	G/G	84
		2	G/A	8
		2	A/A	8
ECS-I γ 2				
Healthy Controls	437	8	G/G	80
		1	G/C	10
		1	C/C	10
	456	3	G/G	30
		4	G/A	40
		3	A/A	30
SLE	437	6	G/G	100
	456	2	G/G	33
		2	G/A	33
		2	A/A	33

DNA sequences across the ECS-I γ 1, ECS-I γ 2 and ECS-I γ 4 regions were determined in SLE and healthy control donors as described in “Materials and Methods Section”, and “Results Section” and in the legend to Fig. 5. The frequencies of ECS-I γ 1 and ECS-I γ 2 polymorphisms did not differ between SLE and healthy control subjects. Only one mutation was detected in the ECS-I γ 4 sequence and is not shown here (see Fig. 5 for location of mutation).