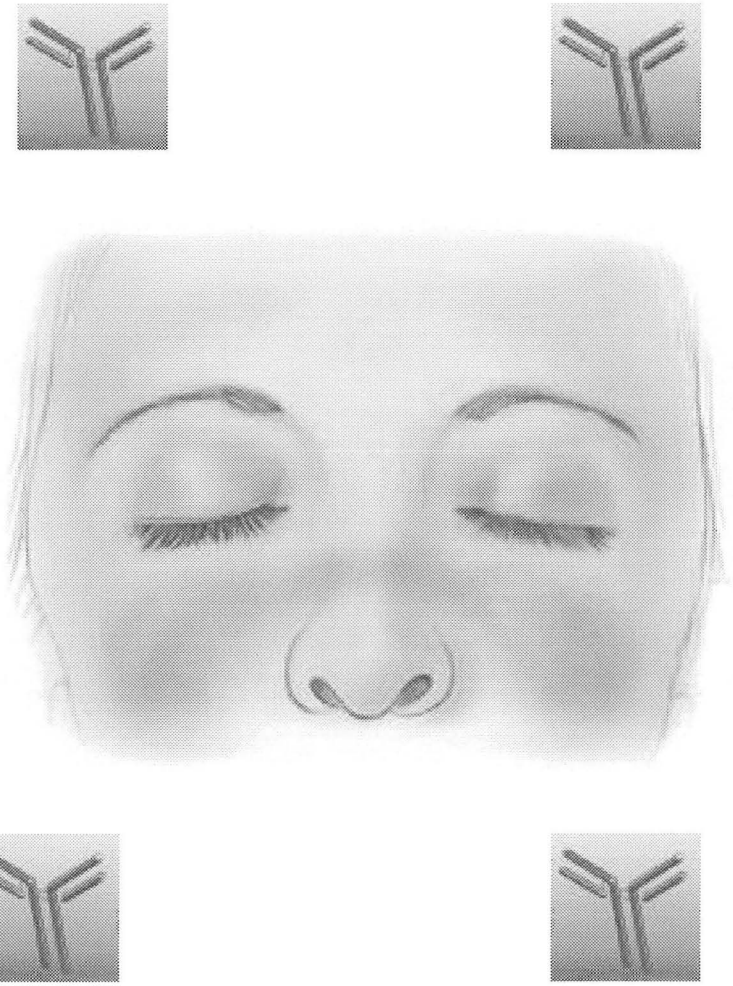
Autoantibodies as Diagnostic Tools and Therapeutic Targets in Systemic Lupus Erythematosus



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Classification

SLE is a systemic disease with prominent autoimmune features. Although clinical features are essential for diagnosing SLE, the American College of Rheumatology has published 11 diagnostic criteria for classification and research purposes (Table below). Four of eleven criteria must be met to diagnose SLE. The criteria serve to standardize the entity of SLE but also leave difficult decisions about patients who have 3 or fewer criteria.

Diagnostic Criteria	Definition
1. Malar Rash	Erythema over malar eminences; usually spares nasolabial folds
2. Discoid lesions	Erythematous patches, keratotic scaling and follicular plugging; atrophic scarring may occur.
3. Oral ulcers	Usually painless, often on hard palate, observed by physician.
4. Photosensitivity	Skin rash as a result of sun exposure
5. Arthritis	Nonerosive, in 2 or more peripheral joints, with redness, swelling, or tenderness. Most commonly at knees, hands, and ankles.
6. Serositis	Pleuritis (pleural pain, pleural rub, or effusion) or pericarditis (by EKG, rub, effusion). Clinically: dyspnea, chest pain or cough.
7. Renal Disorder	Proteinuria >0.5 grams/day or cellular casts (red, hemoglobin, granular, tubular or mixed).
8. Neurologic Disorder	Seizures or psychosis in the absence of offending drugs or metabolic disorder.
9. Hematological Disorder	Hemolytic anemia with reticulocytosis; or leukopenia ($<4000/mm^3$) or lymphopenia ($<1500/mm^3$) on ≥ 2 occasions; or thrombocytopenia ($<100,000/mm^3$).
10. Immunologic Disorder	anti-DNA antibodies, or anti-Smith antibodies (anti-Sm), or positive antiphospholipid antibodies (false +VDRL), positive LE prep
11. Antinuclear Antibody	Abnormal titer ANA (in the absence of drugs known to cause drug-induced lupus)

Tan E.M. et al. Arthritis Rheum 25:1271-7, 1992.

Pathogenesis of SLE

Elements of three main proposed mechanisms describe the involvement of B cells in SLE pathogenesis :

1. Autoantigen presentation (increased availability, abnormal presentation), including abnormal apoptotic clearance.

In SLE, the rate of in vitro PBMC apoptosis is increased due to an increased population of activated lymphocytes (elevated Fas expression, growth factor requirement) rather than a

disease-specific increase in apoptosis [1]. When SLE patients experience a bacterial infection, the rate of in vitro PBMC apoptosis increases further, which correlates with SLE flares after episodes of infection [2].

An elevated rate of apoptosis is not by itself pathogenic, since accelerated turnover of immune cells is a normal part of generating and limiting an immune response. Therefore, the clearance of apoptotic debris has become a major focus of study. SLE patients show reduced clearance of apoptotic debris by macrophages, leading to secondarily necrotic cells, which are removed by an inflammatory response[3]. This slowed clearance is associated with detectable circulating levels of DNA and nucleosomes. Circulating nucleosomes are not pathogenic in patients on hemodialysis, receiving chemotherapy or radiation treatments, but in SLE other immune pathway abnormalities result in immune activation by these stimuli. Cells undergoing apoptosis due to viral infection may present viral antigens together with cellular DNA in apoptotic blebs, leading to epitope spreading and immune responses to normal cellular components [4]. Additional novel epitopes are revealed through the proteolytic caspace cascade that executes apoptosis, and if exposed during necrosis, provides for initiation of an immune response.

2. T cell-dependent B cell activation to make autoantibodies. Autoantibodies in SLE often show evidence of having been created by germinal center reactions: they show isotype switching, affinity maturation, and some emanate from long-lived plasma cells. These features arise from T cell-B cell interactions.
3. Toxicity of autoantibodies (e.g., anti-dsDNA) and immune complexes to end organs as will be discussed below.

These mechanisms combine to form three major effects on B lymphocytes in SLE pathogenesis. First, pathogenic T lymphocytes activate B cells to mature, differentiate, and expand, resulting in increased autoantibody production. Secondly, the skewing of cytokine production favors further B cell growth and differentiation. Finally, the interaction of T cells and B cells through co-stimulatory molecules results in anti-apoptotic signals for selected subsets of the immune system, resulting in the survival and ongoing activity of autoantibody-producing B cell clones [5].

<u>B Lymphocytes in SLE Pathogenesis</u>

Mouse studies have developed several lines of evidence for the importance of B lymphocytes in the pathogenesis of SLE. Most fundamental is that murine SLE does not develop in the absence of B cells [6]. Genetic influences on B lymphocytes in SLE initiation and progression are under active study by multiple groups and have revealed the major contribution of the Sle1 gene in breaking tolerance to nuclear antigens and of the Sle2 gene in permitting increased B cell activation (reviewed by [7]). Finally, manipulation of individual genes has caused altered B cell subset distribution, abnormal signaling, and disruption of normal B cell tolerance. Examples include SHP-1, PD-1, Lyn, CD22, CD19, and TNF family members and their ligands such as BLyS discussed below [8, 9].

B lymphocytes are needed for disease pathogenesis not just because they lead to antibody production, but also because of their effects on activating T cells in a cascade of cytokine production, cell proliferation, and chemotaxis. The JH-MRL lpr -/- SLE mouse model lacks B lymphocytes and therefore also antibodies, but also does not develop the characteristic

lymphadenopathy because of inhibited T cell proliferation [10]. When an IgM transgene is expressed in this model as a membrane protein without producing circulating antibody, adenopathy, T cell proliferation, and lupus organ damage such as nephritis all return. This indicates B cell effects in lupus pathogenesis besides those mediated by autoantibodies. One can speculate that the direct B cell-T cell interaction is critical to causing T cell activation, proliferation, and cytokine secretion, or that B cells cause these effects more indirectly by modulating immune pathways that end in T cell upregulation.

A focus on the pathogenic effects of B lymphocytes in SLE seems counterintuitive given that lymphopenia is commonly seen in SLE, even in patients not treated with steroids or cytotoxic drugs. The explanation is that while overall B cell numbers may be decreased, certain pathogenic subsets are activated and even increased in numbers. Naïve B cells, which do not contribute to antibody production, are reduced in numbers, while the pre germinal center subset (IgD+, CD38+), plasmablasts, and plasma cells are expanded, representing an activated population positioned for antibody production [11]. B cell subsets, such as the VH4.34 cells which do not contribute significantly to the antibody repertoire in healthy controls, frequently form germinal centers and contribute to memory cells and plasma cells in SLE patients, indicating a breakdown in tolerance [12]. Even treatment with immunosuppressives including azathioprine, cyclophosphamide, or cyclosporin did not reduce CD19+CD27+ plasma cell numbers, demonstrating that this cell type is resistant even to aggressive therapy [13].

Plasma cells are the terminally-differentiated B cells that produce antibody in large quantity. Their immediate precursors are plasmablasts, a population that is increased in SLE and retains the capacity to proliferate extensively. Plasmablasts may therefore be sensitive to cytotoxic therapies. Plasma cells can be either short-lived or long-lived. Short-lived plasma cells predominate in most immune responses and can stem from T-independent production in extrafollicular sites, as well as from T-dependent interactions. Long-lived plasma cells have gone through the germinal center reaction and are therefore products of T-dependent responses. It is recognized that some autoantibody titers are not static during the course of SLE, while others change little and do not reflect the disease activity. Anti-dsDNA is the classic example of an autoantibody whose titers frequently rise during increased disease activity, especially for renal involvement. With treatment, the titers may fall again, implying that short-lived plasmablasts and plasma cells are responsible for producing these autoantibodies. On the other hand, the ANA, anti-Sm, and anticardiolipin antibodies do not reflect disease activity and may persist despite successful therapy. This type of antibody likely originates from long-lived plasma cells that are unaffected by current therapies.

Autoantibodies As Clinical Predictors of Disease

Clinical and laboratory features have some value in predicting the severity of SLE. In a study of 245 adult SLE patients over the years 1978 to 2001, Bujan et al. found that cardiac, neurologic, or renal involvement at disease onset leads to a higher risk of subsequent flare in those same organs [14]. Age at diagnosis and valvular heart disease at onset were independent predictors of low survival. Of the laboratory parameters, lupus anticoagulant and aCL antibodies were predictors of stroke. In a different study of 194 SLE patients, Antolin et al. found that SLE that begins before age 20 has a higher association with malar rash (70% vs. 45%), mouth ulcers (48% vs. 29%), and seizures or psychosis (35% vs. 17%) [15].

ANA

The observation of different ANA patterns implies that DNA and histones are not the only targets recognized by these antibodies. The standard screening test for ANAs is immunofluorescence using cultured Hep-2 cells, a human laryngeal carcinoma cell line. Several techniques have been developed to assess sub-components that make up a group of ANAs, often using enzyme immunoassays against recombinant protein or affinity-purified antigen. By using salt-soluble nuclear extracts from calf thymus (extractable nuclear antigens), a further panel of antigens was identified that reacted with some patients' autoantibodies. One of these was the Sm (or Smith) antigen, and others were identified as Ro (SS-A), La (SS-B), and RNP (ribonucleoproteins). Ribonucleoprotein derives its name from its sensitivity to treatment with ribonuclease and trypsin, which cleave RNA and protein.

A large study of ANA tested by indirect immunofluorescence on HEp-2 cell in healthy individuals has provided a baseline of the prevalence of a positive ANA in the population [16]. The frequency of positive ANA did not differ according to age in the range of 20 to 60 years. The findings were of ANA positivity in:

31.7% at 1:40
13.3% at 1:80
5.0% at 1:160
3.3% at 1:320

Therefore, use of a low titer of 1:40 has high sensitivity and low specificity, and captures virtually all patients with SLE, systemic sclerosis, or Sjogren's syndrome. On the other hand, a titer of 1:160 has high specificity and lower sensitivity, and is useful in confirming the presence of disease when there is an elevated pre-test probability, yet excludes 95% of normal individuals. An occasional patient will have a negative ANA but will have autoantibodies to cytoplasmic organelles, such as antiribosome antibodies in SLE or anti-Jo1 antibodies in polymyositis [17].

A negative ANA is a strong indicator of the absence of ANA-related autoimmune disease given the high sensitivity of the test at low titers. Additionally, the predictive value of a positive ANA is low in the primary care setting since the pretest probability of SLE is low [18]. In a review of 1010 consecutive ANA test results in a teaching hospital lab, 153 were positive and resulted in a sensitivity for SLE of 100%, specificity 86% [19]. The positive predictive value for SLE was 11%, and was even lower in patients over age 65, who have a higher rate of ANA positivity. It can be concluded that in populations with a low prevalence of rheumatic disease, such as older patients, the utility of ordering an ANA test is limited but can be improved by restricting its use to patients with a moderate or high pretest probability for the disease.

On the other hand, a group of patients who clinically have SLE or SCLE but a negative ANA on mouse liver has also been described [20]. Such patients uniformly had anti-Ro reactivity (100%), anti-La in 46%, and anti-U1RNP in 35% by ELISA testing. The conclusion was that this pattern of autoantibody formation is actually a definable subset within the spectrum of SLE.

Not only can the presence of a positive ANA have numerous clinical or benign explanations, but the diagnosis of SLE is also based on multiple criteria of which several are clinical. Narain et al studied the diagnoses of 263 patients referred from community practices for evaluation of SLE [21]. 76 patients (29%) had a positive ANA but no clinical disease, and 39 of this subgroup had been treated with prednisone as high as 60 mg/day. 125 of the patients (48%) received a different diagnosis, so that only 23% of presumed SLE was confirmed upon rheumatologic consultation.

A positive ANA is a frequent cause of referral for rheumatologic consultation. In considering the prevalence of autoantibodies in the community, Fernandez et al. tested for ANAs in 500 normal blood donors [22]. ANAs were detected in 22.6% of subjects, most at a low titer of 1:40. In a report of 93 adult Malaysian blood donors, ANA was found in 6.5%, anti-dsDNA was not found, and anti-Sm and anti-RNP were each seen in 1.1% [23]. Elderly individuals have an increased incidence of positive ANA titers. In one study of 64 healthy men and women with a mean age of 81, 31.3% had a positive ANA, while anti-Ro was 1.6% positive, anti-dsDNA in 14.1%, and anti-cardiolipin IgG was 51.6% [24]. A comparison group of 261 non-elderly revealed only aCL antibodies, in 2.3%.

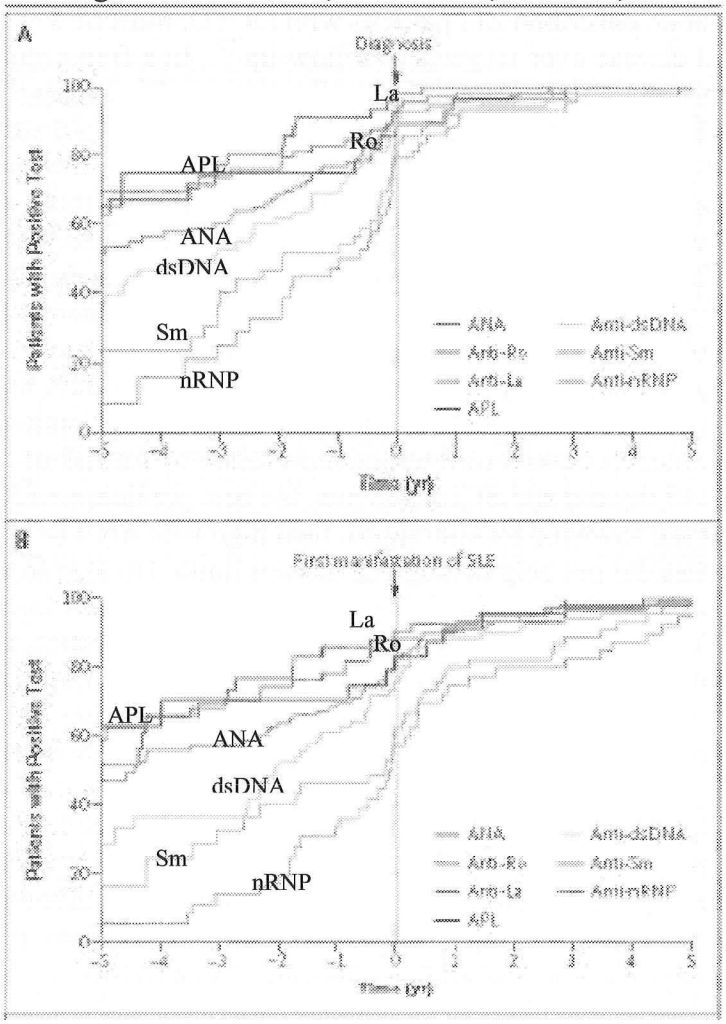
ANA positive patients that do not meet criteria for a specific rheumatic disease (also called undifferentiated connective tissue disease (UCTD)) have been followed in series of up to several hundred cases. Williams et al. described 213 patients with UCTD, most of who continued to have undifferentiated disease over 10 years of follow-up [25]. In a Dutch study, 65 ANA+ patients without anti-dsDNA antibodies were followed for a mean of 9.3 years [26]. Of these 38 (58%) developed a specific rheumatic disease (most commonly rheumatoid arthritis (17), Sjogren's syndrome (12), SLE (6), osteoarthrosis (3)). Five developed non-rheumatologic conditions and 22 remained undiagnosed but developed no severe symptoms. In those diagnosed with a condition, the diagnosis was apparent in 75% by 2 years, and in 90% by five years. In a further series, 91 patients with UCTD and at least 1 non organ-specific autoantibody were followed for one year or longer [26]. Clinical symptoms that were inversely correlated with development of SLE on multiple regression analysis were sicca symptoms, Raynaud's phenomenon, and photosensitivity. Of those who developed no serious disease, 82% had a single autoantibody (30% anti-Ro alone, 28% anti-RNP alone). In another study, patients with a limited form of lupus with predominantly cutaneous findings were followed for risk of developing systemic disease. In 245 discoid and SCLE patients, the best predictors of SLE were nephropathy (proteinuria, hematuria), followed by arthralgias, then high titer ANA (>1:320) [27]. Interestingly, anti-dsDNA antibodies did not help distinguish disease limited to skin from SLE.

Incomplete lupus erythematosus (ILE) can be defined in patients with fewer than 4 of the 11 diagnostic criteria for SLE. When incomplete lupus is limited to one organ system, it has an excellent prognosis, with only 3 of 100 patients developing SLE during 3 years of follow-up [28]. Not surprisingly, ILE patients are most likely to show less serious features, positive ANA, nonerosive arthritis, and cutaneous involvement [29]. In a study of 28 Swedish patients with some clinical features of SLE but fewer than 4 ACR criteria, 16 patients (53%) developed complete SLE in a median of 5.3 years [30]. Only 1 of 3 patients with anti-dsDNA antibodies developed SLE, 6 of 6 patients with malar rash progressed to SLE, and 3 of 8 patients with arthritis developed SLE. This study followed patients who had at least one organ system involved and were felt clinically to likely have SLE, therefore representing the more severe spectrum of incomplete SLE. At the less severe end of the spectrum are patients with a positive ANA but fibromyalgia, who were distinguished as less likely to be African-American, and clinically showed less mucocutaneous and hematologic involvement than SLE patients [31].

The ANA titer does not correlate with changes in disease activity, and the ANA continues to be present even when clinical SLE is apparently inactive. In following 14 patients with SLE inactive for a mean of 8.9 years (range 3-22 years), one study found that the ANA remained positive in 71.5% of cases, with ENAs in 14.3%, but no anti-dsDNA [32]. Therefore, ongoing monitoring of SLE patients even with clinically inactive disease is recommended.

Autoantibody development before clinical disease onset

A recent large retrospective study provides new insight into the development of autoantibodies before the clinical diagnosis of SLE [33]. Using serum samples stored by the US military on its members, 130 SLE patients and 4 matched controls per patient were identified who had serum samples collected before the diagnosis of SLE. There was a large difference between the presence of autoantibodies in SLE patients versus controls, before clinical SLE developed: ANA (at 1:120 dilution) in 78% of patients, 0% in controls. Multiple other antibodies were also found at higher levels in patients: anti-Ro, anti-La, anti-Sm, anti-RNP, anti-dsDNA, antiphospholipid (figures below). Antibody levels in controls agreed with previous studies, showing 3% anti-dsDNA, 3% anti-Ro, 2% APL, and 2% anti-RNP.



Autoantibodies before SLE diagnosis.

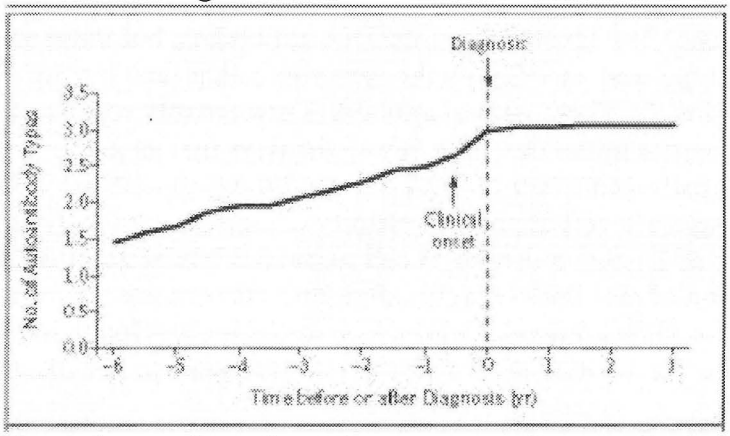
Autoantibodies before first manifestation of SLE..

Serum had been stored as far back as 1985, allowing an estimate of the interval between initial antibody development and the onset of SLE. Because 90 of 130 (69%) of patients had autoantibodies in the first available sample, the analysis represents an underestimate of the true values. The findings were that at least one autoantibody was present a mean of 3.3 years before diagnosis in 88% of patients. Three distinct groups of antibody formation were described: 1. ANA, antiphospholipid, anti-Ro, and anti-La developed a mean of 3.4 years before diagnosis; 2. anti-dsDNA formed a mean of 2.2 years before diagnosis; and 3. anti-Sm and anti-nRNP developed 1.2 years before diagnosis. In the group as a whole, new types of autoantibodies

accrued steadily in the 6 years prior to diagnosis, increased the year before diagnosis, then remained stable thereafter. In part, this reflects the high rate of positivity for the autoantibodies seen by the time of diagnosis. However, this accumulation of autoantibodies does not imply a stable disease pattern. It has been recognized clinically that SLE patients may develop additional autoimmune diseases over time, most commonly Sjogren's syndrome, rheumatoid arthritis, autoimmune thrombocytopenia, antiphospholipid syndrome, and hypothyroidism [34].

It was apparent that patients developed individual clinical criteria of SLE at times more than 10 years before formal diagnosis. 16% developed a clinical symptom more than 3 years before diagnosis, and only 21% presented with their first clinical symptoms in the month of SLE diagnosis. In addition, autoantibodies developed before clinical manifestations in 90% of the SLE patients. The ANA, anti-Ro, anti-La, APL, and anti-dsDNA formed well before the initial clinical manifestations of SLE, while only anti-Sm and anti-nRNP coincided with the clinical diagnosis.

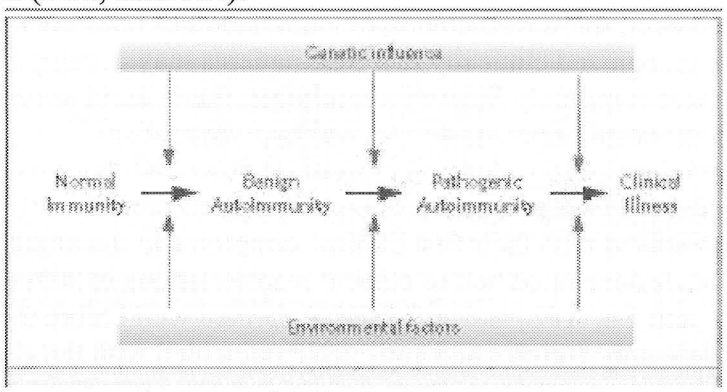
This study is valuable in its examination of a large number of samples in the years before SLE becomes clinically apparent. It has provided new insight into the sequence of autoantibody induction and identifies groups of antibodies and has allowed the postulation of a period of benign or subclinical autoimmunity, followed by pathologic autoimmunity and finally fully-developed clinical disease. It has been previously observed that the autoantibody types seen years before SLE (ANA, anti-Ro, anti-La, APL) also occur in many normal subjects and are often not associated with disease. Similarly, anti-dsDNA, anti-Sm, and anti-nRNP are more closely associated with clinical SLE and are rarely seen in normal people. Drawbacks to the study are that is a retrospective review relying on chart notes for clinical evaluation. A long-term prospective study of subjects at risk for developing SLE would address this concern. As the data here show, some of the subjects with asymptomatic autoantibody formation or subjects with incomplete SLE will progress to frank SLE over a number of years. While this study deals only with patients that went on to develop clinical SLE, a much larger study is needed to define which laboratory and clinical feature predict progression in those with asymptomatic autoantibodies or minimal clinical findings.



Autoantibody accumulation before SLE diagnosis.

These observations about the sequence of autoantibody induction also have implications for theories of SLE pathogenesis (reviewed in [18]): If environmental triggers of SLE exist, these may occur many years before disease onset. Given the accrual of autoantibodies, it may even be necessary for a sequence of triggers to induce the different classes of autoantibodies. While epitope spreading has been well-described for some autoantibody systems (e.g. the anti-Sm response), it remains possible that the initial induction of less pathogenic or disease-specific

autoantibodies (ANA, aPL, aRo, aLa) are involved in more disease-specific autoantibody formation (aSm, adsDNA).



Phases of autoimmunity in SLE.

Genetic Factors and Autoantibodies

Male African-American SLE patients have been found to have the most rapid progression from the first clinical symptoms to the diagnosis of SLE [35]. The median time to diagnosis was shorter in AA males compared to AA females, or European-American males or females. In addition, African-American males were more likely to have nephritis as their first clinical symptoms, heralding a severe form of SLE.

Genetic factors are also being studied to explain the multiple clinical associations in families with SLE sib pairs: thrombocytopenia, discoid rash, neurologic disorder, hemolytic anemia, age of SLE diagnosis [36]. However, because the average time between dates of diagnosis was 11 years in parent-offspring pairs and 7.5 years in affected sibpairs, it is likely that shared immediate environmental triggers are not the explanation for SLE induction. Genetic influences also play a major role in autoantibody profiles. In four sets of identical twins discordant for SLE, 3 of 4 pairs had virtually identical anti-RNA protein (Ro, La, Sm, U1RNP) antibodies and anti-RNA antibodies, although the affected sib had higher titers [37]. Such studies demonstrate that genetic factors are important in determining autoantibody profiles.

Anti-double stranded DNA (dsDNA) antibodies

Even healthy controls produce low levels of anti-dsDNA antibodies, but these are generally low-affinity, of IgM isotype, and encoded by the germline rather than having undergone a germinal center reaction [38]. These natural antibodies presumably represent part of the innate immune system that provides initial defenses during the days that an adaptive immune response develops. However, the pathogenic anti-dsDNA autoantibodies of SLE are IgG, high-affinity antibodies that have undergone T cell-dependent affinity maturation and isotype switch recombination. The essential role of T cells in driving B cell responses has been reinforced by the experimental in vitro production of anti-dsDNA antibodies after stimulation with histone-specific T cells [39]. It is likely that several antigenic epitopes on nucleosomes, including histones, other proteins, and modified DNA, will be identified over time to contribute to anti-dsDNA induction.

Evidence exists for multiple ways in which anti-dsDNA antibodies cause disease. The antibodies can bind directly to targets, including heparan sulfate, the major glycosaminoglycan of the glomerular basement membrane, damaging the kidney [40 41]. The cellular structures that bind anti-dsDNA antibodies now include: Fc receptors, Hp8 on renal tubular cells, ribosomal P proteins on glomerular mesangial cells, myosin 1 in rat hepatoma cells, and calreticulin on the cell surface. Several of these interactions contribute to inflammation by causing an increase in

cytokines, including IL-1, IL-6, IL-8, TGFβ, and von Willebrand factor (reviewed in [42]). Anti-dsDNA may interact with cell surface molecules but have also been shown to penetrate living cells in culture to bind ribosomal protein S1, with potentially toxic consequences. [43]. When injected into mice, human monoclonal anti-dsDNA antibodies deposited in the glomeruli, mesangium, and capillary walls, causing proteinuria [44]. Anti-dsDNA antibodies also formed soluble histone-DNA-anti-DNA immune complexes that can deposit in glomerular capillary wall [45]. Therefore, anti-dsDNA antibodies may cause disease by direct binding, by forming immune complex that deposit in the kidneys, and by cross-reacting to cellular antigens.

Antibodies to dsDNA and the Sm antigen are not strictly pathogenic in every case, since low levels have been described in IgG isolated from normal subjects [46]. It was found that IgG could be affinity-purified from immunoabsorbent columns of human $F(ab')_2$-Sepharose to give antibody that reacted with $F(ab')_2$ but also with dsDNA and Sm. This antibody represented 0.02% of normal serum IgG and was also seen in SLE patients. The mechanistic implication is that a network of idiotypic-antiidiotypic interactions exists even in normals, but is perturbed in SLE patients to allow the expansion of pathologic antibody specificities.

Anti-DNA Autoantibodies are the Source of Further Immune Stimulation.

Anti-DNA antibodies can be induced by nucleosomes, bacterial DNA, but also by fragments of the anti-DNA antibodies themselves (reviewed in [47]). Epitopes from the variable regions of anti-DNA antibodies can be effective stimulators of T-lymphocyte responses, as seen in human SLE and in the (NZB x NZW)F1 mouse model of SLE. In a recent study, PBMCs from 31 SLE patients and 20 matched healthy controls were evaluated for cytokine responses after treatment with seven peptides from anti-DNA antibodies. In relatively early SLE, significantly more secretion of IFNγ, IL-4, and IL-10 occurred in SLE samples than controls, indicating a mixed Th1 and Th2 response. In late disease, Th2 cytokines predominated. Interestingly, fewer peptides elicited responses in late disease, raising the possibility of clonal exhaustion, deletion, or regulation. In animal models, peptides from anti-dsDNA antibodies have been used in high doses to induce tolerance to this protein antigen. The treatments result in delayed onset of disease, delayed disease progression, and prolonged survival [48, 49]. Based on these observations, there is interest in using one or more such peptides in treating human SLE.

In MRL/lpr and MRL/+ mouse models, anti-dsDNA antibodies (detected by Farr assay) precede the development of anti-nucleosomal antibodies [50]. The anti-dsDNA antibodies showed anti-nucleosome activity as well, but this could be completely inhibited by the addition of free dsDNA. Therefore, the anti-nucleosomal activity was entirely due to cross-reactivity before antibody specific to nucleosomes developed later. Other studies in mice have demonstrated that histone-containing immune complexes are largely responsible for the anti-dsDNA reactivity of antibodies in the Farr assay [51]. Farr reactivity of purified IgG preparations could be restored by adding purified histones. Interestingly, histones can be demonstrated in the serum and plasma of SLE patients, but not in healthy controls.

Clinical Implications

The presence of anti-dsDNA has low sensitivity and moderately high specificity in SLE. Sensitivity is limited because only 50 to 60% of SLE patients will show anti-dsDNA antibodies at some point in their course, while specificity for SLE is affected by the occurrence of anti-dsDNA antibodies in cases of autoimmune hepatitis, syphilis, bacterial endocarditis, parasitic and other infections [42]. The performance characteristics of the anti-dsDNA assay can also affect

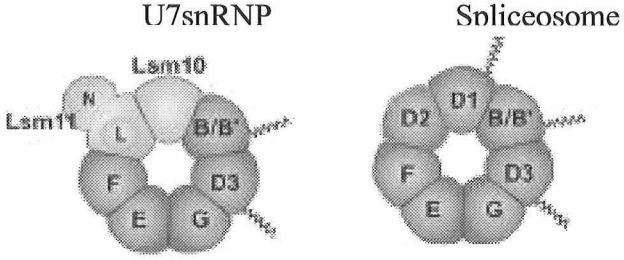
results, as an ELISA was found to be positive in 30% of ANA-positive subjects without SLE [52]. Immunoprecipitation by Farr assay and immunofluorescence on the kinetoplast of Crithidia luciliae are more commonly used. The Crithidia assay has the highest specificity since no histones or single stranded DNA are present to confound the results.

In a study of stored samples from members of the military, 130 SLE with 633 serum samples were analyzed for anti-dsDNA Ab by ELISA [53]. 55% of subjects had anti-dsDNA Ab before SLE diagnosis, appearing at a mean of 2.7 years before diagnosis (range 9.3 years to less than 1 month). Of 26 cases with at least 2 positive samples, there was a rise in antibody titers within 6 months of diagnosis. Those with anti-dsDNA Ab at diagnosis were more likely to have renal disease (66.7% vs. 27.3%). The 7 cases treated with steroids had a significant drop in antibody titers. This study shows that even anti-dsDNA antibodies can be present for years before SLE can be diagnosed, and that a rise in titer may herald a more active phase of the disease. It does not address the issue of how many patients with ANA and anti-dsDNA antibodies are not destined to progress to overt disease.

ENA (Extractable nuclear antigens)

ENA derive their name due to their extraction by phosphate-buffered saline from calf thymus. The Sm and RNP antigens are ribonucleoprotein particles involved in the splicing of precursor mRNA.

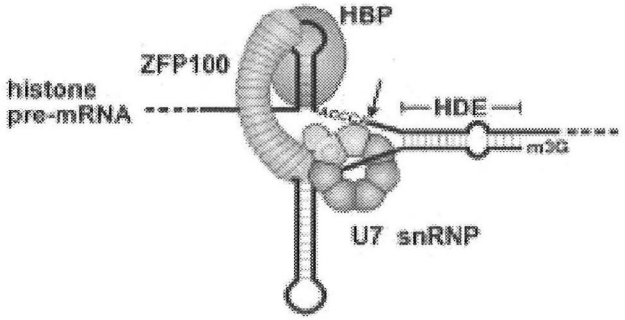
The ENA panel consists of anti-Smith (Sm), anti-RNP, anti-Ro (SSA), and anti-La (SSB) specificities. The clinical utility of adding this testing to an ANA has been addressed. In a review of 2185 samples tested for ANA, 259 were positive and chart reviews were performed without knowledge of ENA results [54]. Univariate and multivariate analysis were used to determine the utility of ENA in the diagnosis of SLE. In this limited panel, anti-SSA had a strong predictive diagnostic value among ANA+ and anti-dsDNA negative patients (but added no new information in those who were already anti-dsDNA positive), and overall positivity in the ENA panel correlated with more pleuritis. However, no major clinical differences could be found. More detailed studies exist for clinical associations of individual ENA components.



Anti-Sm

Sm (Smith) antigens are a subset of the group of common core proteins that complex with small nuclear RNAs (called U1, U2, U4-6, U5) to form small nuclear ribonucleoprotein particles (snRNPs) (reviewed in [55]). Seven different core proteins form heptamer rings and snRNA passes through the center of the structure. The function of these structures is the splicing of precursor messenger RNA to remove introns or to generate alternatively-spliced mRNA [56]. The B, D1, and D3 core proteins are the major Sm epitopes. Because multiple core proteins act as antigenic stimulus forming multiple anti-Sm antibodies, reactivity to Sm has been described as an antibody system rather than a single entity [57]. The anti-RNP antibodies described below react with the U1 RNA and its associated proteins 70K, U1-A, and U1-C.

Anti-Sm antibodies are found in approximately 30% of all SLE patients but are highly specific. In a comparison of complement fixing capability among ANAs, it was found that 18 of 20 SLE patients had ANA that could fix complement, while only 2 of 18 drug-induced ANAs could [58]. Complement-fixing ANAs were significantly correlated with anti-Sm activity, as well.



mRNA for Splicing

U7 snRNP

Anti-Sm response and EBV infection

Previous EBV exposure is more common in SLE patients that in controls [59]. In comparing 196 adult ANA-positive SLE patients with 392 age-, race-, and sex-matched controls, the SLE patients had a significantly higher EBV exposure (99.5% SLE vs. 94.4% control, p=0.014). In pediatric cases, 116/117 SLE patients had seroconverted against EBV, compared to only 107 of 153 controls (OR 49.9). Exposure to other viruses (CMV, HSV1, HSV2, VZV) was not different. These observations raise the possibility that an immune response against EBV may be misdirected against human sequences, leading to autoimmunity.

In comparing human autoantibody targets with viral sequences, a stretch of the Sm B protein was found to have a high degree of similarity to the EBV major DNA binding protein (EBNA-1) (reviewed in [60]). The EBNA-1 epitope PPPGRRPFFHPVGEA has significant overlap with the first epitope in an anti-Sm response, PPPGMRPP [61]. The antibody response to this EBNA-1 epitope is not present in EBV-seropositive individuals but instead is temporally restricted to an acute EBV infection. The opportunity to initiate a cross-reactive immune response is therefore not open-ended. There is a similar or identical stepwise progression in the early humoral immune maturation of anti-Sm, with initial reactivity to the repeated, proline-rich sequence of SmB, PPPGMRPP, and subsequent use of the second epitope, PPPGMRGP [62]. Further epitope enlargement occurs by incorporation of neighboring amino acids. The sequence of events is nearly identical in different lupus patients. In animal models, immunization with the peptide PPPGMRPP leads to antispliceosomal autoimmunity and feature of lupus, with spreading of the humoral immune response to produce ANA, anti-nRNP, and anti-dsDNA [63, 64]. This work was recently extended by the observation that immunization of animals with either of two antigens produced similar laboratory and clinical finding: the first epitope of 60 kDa Ro or the cross-reactive EBNA-1 epitope both caused autoantibodies to Ro and to other spliceosomal autoantigens, and eventually resulted in clinical leucopenia, thrombocytopenia, and renal dysfunction [65]. This line or research has demonstrated molecular mimicry between viral antigens and cellular components as well as a consistent sequence of epitope spreading as part of autoimmunity induction.

Sm **PPPGMRPP**

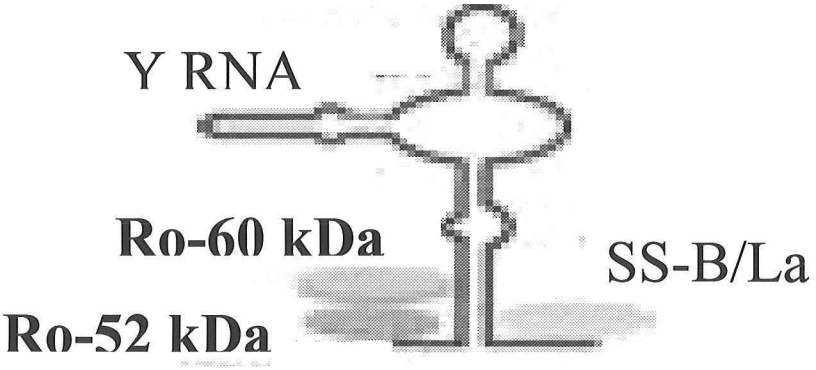
EBNA-1 **PPPGRRP**FFHPVGEA

Anti-SSA (Ro), Anti-SSB (La)

Anti-Ro is targets extractable nuclear antigens and recognizes epitopes associated with small RNAs of unknown function known as Y RNA, located in the cell's cytoplasm or nucleus. The Ro protein also binds to misfolded 5S rRNA precursors, leading to a theory that Ro protein is involved in recognition or repair of intracellular damage, especially in the ribosome biogenesis pathway. The Ro proteins are divided into 2 major forms: 52 kD Ro, containing subtypes α and β, and 60 kD Ro. Mice lacking the 60kD Ro protein develop a lupus-like syndrome including glomerulonephritis, photosensitivity to UV light in some strains, and autoantibodies to ribosomes and chromatin [66]. The 52kD Ro protein is encoded by a separate gene and is clinically associated with atrioventricular heart block and interruption of L-channel calcium influx into cardiac myocytes in neonatal lupus.

The single La protein is 48 kD. Early work proposed that the function of La is to act as a termination factor for RNA polymerase III, as it binds to the 3' end of virtually all nascent polymerase III transcripts [67]. More recent studies of the cell cycle demonstrated that La protein prolongs the half-life of histone mRNA, potentially leading to increased histone protein production [68]. An additional function for La protein has now been described in the maturation of pre-tRNA species [69]. La protein is the first protein to interact with pre-tRNAs and prevents further maturation of the tRNA until La is specifically phosphorylated. Clinically, La is associated with SLE, with neonatal SLE, and with Sjogren's syndrome.

The technique used to detect autoantibodies has been especially critical in the detection of anti-Ro. In a study of 4025 consecutive serum samples tested for ANA, 285 (7%) were positive for Ro reactivity by ELISA [70]. However, 75 (26%) of the Ro-positive samples were negative on ANA screening by immunofluorescence on Hep-2 cells, including 12 patients with confirmed SLE. Additional testing can now be performed using Hep-2 cells overexpressing transfected 60kD Ro antigen (Hep-2000(R) cells), by immunodiffusion, and by Western blot, but there continue to be cases only detected by the sensitive Ro ELISA assay. In addition, both anti-Ro and anti-La antibodies contain subpopulations of antibodies to idiotypes on anti-dsDNA, thus masking and possibly down-regulating anti-dsDNA antibodies [71].



Clinical Associations for Ro and La

Anti-Ro responses likely result from processing and presentation steps similar to that for a foreign antigen, since IgM antibodies form first, followed by IgG anti-Ro antibodies [72]. Furthermore, there is evidence of a germinal center reaction in the anti-Ro response, as the antibodies increase in complexity and affinity over time. Normal subjects can have detectable

levels of anti-Ro (in 18% of 40 samples) and anti-La (8% of 40 samples), although titers were 1000 fold lower than the highest levels measured in patients [73].

Anti-Ro and anti-La are found most commonly in patients with SLE, Sjogren's syndrome, systemic sclerosis, neonatal lupus, and others. Within the range of lupus categories, the anti-Ro response has been associated with SCLE, neonatal lupus, drug-induced lupus, ANA-negative lupus, and the lupus-like syndrome associated with homozygous deficiency of complement components C2 or C4 [74]. Anti-Ro responses are seen in about 30% of patients with SLE and in 90% of elderly onset SLE (above age 50) [75]. A recent large-scale study was performed to quantitate the clinical associations of Ro and La [76]. The investigators determined the ANA in 10,550 consecutive patients, and positive samples were tested for reactivity with recombinant Ro52, natural Ro60, and recombinant La. Of those with positivity of anti-Ro and/or anti-La, clinical information was available on 181 patients. The associated diseases were: SLE in 45.3%, primary Sjogren's syndrome in 14.4%, progressive systemic sclerosis in 8.8%, rheumatoid arthritis in 7.7%, cutaneous lupus in 7.7%, and dermatomyositis in 2.2%. The presence of Ro52 antibody alone enriched for systemic sclerosis (34.2%) and dermatomyositis (10.5%). Anti-Ro60 indicated the presence of SLE (80%), as did Ro60 plus Ro52 reactivity (SLE in 52.2%). When Ro52, Ro60, and SSB were detected in the same sample, it signaled SLE in 55.8% and systemic sclerosis in 20.9%. The unusual pattern of anti-La alone indicated systemic sclerosis (33.3%), cutaneous lupus (23.8%), but systemic lupus in only 14.3%. A further study of Ro60, Ro52, and La antibodies in 18 Ro-positive SLE patients followed over 18 to 44 months, repeated testing demonstrated that the antibody profile for these three antigens is fixed early in disease and hardly changes in most patients [77].

To determine the prognostic significance of anti-Ro reactivity, 100 anti-Ro positive patients were followed for 10 years [78]. The initial diagnoses were: SLE (51%), Sjogren's syndrome (17%), SLE/Sjogren's overlap (7%), UCTD (16%), others (9%). After 10 years, 65% (51 of 78 evaluable patients) had a progressive disease process: Sjogren's syndrome (7 of 17 patients), SLE (23 of 51 patients), SCLE, renal disease (24%), rheumatoid-like arthritis (in 17 patients). Severe manifestations of rheumatic disease, interstitial lung disease, CNS disease, and vasculitis, were notably frequent. African-Americans had earlier disease onset and a more severe course compared to whites. Additionally, cutaneous manifestations were common in Ro^+ SLE patients: photosensitivity, malar rash, discoid lesions, SCLE lesions.

Anti-La has recently been identified as an anti-neutrophil autoantibody that may be responsible for neutropenia in SLE [79]. About 20% of SLE patients were found to have such anti-neutrophil antibodies by ELISA testing. Human anti-La antibodies penetrate into normal human neutrophils, suppress phagocytosis, accelerate apoptosis, and enhance IL-8 production. These mechanisms could account for neutropenia and functional impairment of neutrophils in SLE.

Anti-RNP

RNP or ribonucleoproteins was originally described as a combination of RNA and protein sensitive to degradation by ribonuclease and trypsin [80]. This distinguished it from the Sm antigen, which was resistant to such treatment. The U1 snRNP is the target of anti-RNP antibodies, and within this structure, three main antigens have been described: 70K protein is the most common, and the U1-A and U1-C proteins are secondary. Additionally, 60% of anti-RNP-positive patients have IgM or IgG anti-U1 RNA antibodies, and all of these had anti-70K and most had anti-U1-A [81]. The clinical association of anti-U1 RNA positive patients was of increased incidence of HLA-DR2/DR4, Raynaud's phenomenon, and synovitis. Therefore, the

commonly-tested anti-RNP antibodies are directed at three protein antigens, but antibody to the U1 RNA itself is also present and has distinct clinical associations.

The U1RNP antibody is an essential part of diagnosing mixed connective tissue disease (MCTD) but is present in about 30% of SLE patients. A recent study of 235 SLE patients included in-depth analysis of antibodies to RNP-70K, RNP-A, and RNP-C [82]. Not surprisingly given the proximity of relevant antigens, patients had a clustering of antibodies to SmB, SmD, RNP-70K, RNP-A, and RNP-C. Clinically, Raynaud's phenomenon was associated with antibody to all three RNP proteins, while leucopenia was seen in those with anti-RNP-70K and anti-RNP-A. A lower incidence of urinary cellular casts was found in those with anti-RNP-A and anti-RNP-C.

Like anti-dsDNA antibodies, anti-RNP antibodies have been shown to penetrate cells [83]. Therefore, their mechanism of action may include alteration of normal cellular functions, even the induction of cellular death by apoptosis or necrosis, thereby contributing to ongoing exposure of RNP structures as antigenic stimuli.

Antiphospholipid antibodies

Given the above studies of autoantibody accumulation prior to clinical diagnosis of SLE, the presence of APL antibodies has been investigated in individuals at risk of lupus. Multiple sera were available for analysis before and after onset of SLE in 130 patients and were tested for IgG and IgM aPL by ELISA [84]. (This study utilized the same military repository of stored serum samples as the study by Arbuckle et al. discussed on page 7). The results showed 18.5% of cases had IgM and/or IgG aCL before onset of clinical SLE, at 7.6 years to 1 month (mean 3 years) before diagnosis. The presence of anticardiolipin antibodies early in the course of SLE predicted a more severe clinical phenotype, with the presence of 6.1 of 11 SLE diagnostic criteria, versus 4.9 in other SLE patients. The ACL positive patients had more frequent renal disease, CNS disease, thrombocytopenia, and clotting events.

Other assays.

With over well over 100 antigen-autoantibody pairs already described in SLE [85], there will be ongoing attempts to find laboratory values that predict the onset, worsening, or response to treatment in SLE. Among newer assays, anti-C1q has been used for monitoring, not diagnosis; anti-nucleosome antibodies are used for diagnosis and monitoring, and urinary levels of MCP-1 have been studied to follow lupus nephritis [86]. Proteomic approaches may allow the collection of peptides from most known lupus antigens (and multiple other candidate antigens) onto a single chip. One can envision a leap forward in diagnostic associations when an individual's serum can be tested in this comprehensive manner.

Treatments

SLE is treated by long-established medications such as corticosteroids and hydroxychloroquine, which are not curative. The standard of care for proliferative lupus nephritis is currently the use of intravenous cyclophosphamide plus corticosteroids, following a protocol first studied at the NIH. Common toxicities from such treatment include diminished resistance to infection from cytopenia, gonadal toxicity [87], steroid side effects, and even a long-term risk of malignancy. Since all rapidly proliferating cells can be affected by a cytotoxic

regimen, not only inflammatory cells but mucosa and bone marrow are common locations for unintended side effects.

Targeting of the B lymphocyte lineage as a treatment for active lupus would have the benefit of avoiding nonspecific killing of all rapidly proliferating cells and leaving significant mucosal and cell-mediated immunity to maintain microbial resistance. The approach would have clinical utility if the B lymphocyte lineage plays a major role in lupus pathogenesis, and if eliminating this single branch of an immune response controls the pathogenic immune reactivity.

Non-specific reduction of inflammation: Glucocorticoids

Glucocorticoids represent the main rapidly-acting medication available to treat severe manifestations of SLE. In an inception cohort of 73 patients followed for at least 15 years, Gladman et al. found that prednisone was used by 87.7% of patients, for a mean of 117.1 months, i.e. almost 10 years [88]. Organ damage seen over time in SLE patients was from the disease itself early on but was felt to be mainly from steroid use when assessed at year 15.

Glucocorticoids exert their main effects by binding to the glucocorticoid receptor, a member of the family of nuclear receptors, which act as ligand-dependent transcription factors. The human glucocorticoid receptor α (hGRα) is widely expressed on almost all tissues and cells, making glucocorticoid effects potentially universal in their scope. Specificity of action is achieved by multiple mechanisms, including access to the multiprotein complex containing the glucocorticoid receptor in the cell's cytoplasm, access to the promoter regions of specific target genes in the cell's nucleus, protein-protein interaction with other transcription factor families (NF- κB, AP-1, STAT), and interactions with coactivators and corepressors that modulate the transduced signal (reviewed in [89]). Recently-described non-genomic effects of glucocorticoids cause shifts in membrane structures or organization, with consequences such as altered calcium responses after stimulation [90].

Many aspects of immunity are inhibited by glucocorticoids. Innate immunity is downregulated, with dampening of pro-inflammatory cytokines (e.g. IL-1, TNFα, IFNγ, IL-12) and upregulation of anti-inflammatory actions (e.g. IL-10, TGFβ, IL-1RA). Adaptive immune responses are pushed to the Th2 response, away from Th1 responses. The blood leukocytosis frequently seen with corticosteroid use promotes survival and proliferation of neutrophils; enhances GM-CSF effects, enhances LTB4-mediated neutrophil survival and chemotaxis, increases the release of bone marrow neutrophils, and inhibits neutrophil transmigration to inflammatory sites by inhibiting leukocyte adhesion molecules such as L-selectin. The immediate effect on neutrophils is to cause shedding of L-selectin. Later effects include inhibition of release of IL-8 and other CXC chemokines.

Given the substantial evidence that glucocorticoids allow or promote a humoral immune response driven by a Th2 phenotype, what is the effect on immunoglobulin production? The research in this area is less robust, but certain immunoglobulin isotypes can actually be elevated after glucocorticoid treatment, such as IgE in asthma. This effect may be balanced in part by the observation that earlier steps in B cell proliferation and development are downregulated by glucocorticoids. However, Ig-secreting plasma cells have become largely resistant to the inhibitory effects of glucocorticoids. One can speculate that autoimmune or inflammatory diseases are not worsened by glucocorticoids because of their other anti-inflammatory effects: elimination of effector cells (mast cells, eosinophils, basophils), reductions in proinflammatory cytokines, and less chemotaxis of inflammatory infiltrates. In addition, elevations in immunoglobulin levels do not necessarily represent antigen-driven specific responses

Apoptosis

High doses of corticosteroids lead to B cell, T cell, and monocyte apoptosis, representing an end to proinflammatory effects as long as the apoptotic debris is cleared efficiently. Neutrophil apoptosis is delayed after corticosteroids administration, yet corticosteroids also increase the capacity of macrophages to clear apoptotic debris [91].. Within the B lymphocyte lineage, pro- and pre-B cells are most sensitive to apoptosis, with less sensitivity in more mature forms [92, 93]. The clinical effects of glucocorticoid treatment are not long-lasting, as lymphocyte repopulation from progenitors is rapid and complete [94].

Summary of corticosteroids effects:

In autoimmune diseases, corticosteroids are used because they are a widely-available, rapidly acting modulator of the immune system. Their main effects are the elimination of significant parts of the B- and T-lymphocyte compartments. Also important are a reduction in pro-inflammatory cytokines and chemokines, with attendant reduction in inflammatory infiltrates and a skewing of cytokine responses to favor Th2 rather than Th1 cells. Glucocorticoids diminish the stimulation of T cells by dendritic cells, leaving a more immature dendritic cell population that favors generation of suppressor T reg cells, and tolerance.

Targeting of B Lymphocytes

Anti-CD20

CD-20 is a membrane-associated glycoprotein expressed on pre-B cells, resting B cells, and activated mature B cells, but not on pro-B cells, most memory B cells, and terminally-differentiated plasma cells. The function of the CD20 molecule is not well-understood, as CD20-deficient mice have no evident immunologic phenotype [95]. When used as a therapeutic target, CD-20 has the advantages of being stably expressed on the cell surface without internalization or shedding even after anti-CD20 binding. Binding of anti-CD20 antibody to CD20+ cells causes cell death without the need for an additional toxin.

Rituximab has been an FDA-approved therapy for relapsed or refractory, low-grade B cell follicular non-Hodgkin's lymphoma since 1997, with over 370,000 doses given. Rituximab is an anti-CD20 humanized, chimeric monoclonal antibody. Its antigen combining site consists of murine anti-human CD20 sequence and is fused to human IgG and κ constant regions. Efficacy of rituximab depends on the presence of a high-affinity allele of the Fcγ receptor IIIa, with lower B cell depletion and diminished clinical efficacy seen when the low-affinity allele (F176) is present [96, 97]. Homozygosity for the low affinity allele FcγRIIIa and African-American ancestry are associated with a failure to deplete [98].

B cell depletion after rituximab reaches > 99% when effective and generally lasted 6 to 9 months in lymphoma trials. Since long-lived antibody-secreting plasma cells do not express CD-20 and are therefore not eliminated by rituximab, circulating levels of IgG and IgA are generally unaffected by rituximab treatment. A modest drop in IgM levels has occurred in some, possibly reflecting a diminished ability to generate an antibody response that requires the maturation and differentiation of B lymphocytes. With these modest changes in antibody levels, a higher rate of infectious complications has not been seen in lymphoma trials [99]. The effect of rituximab on the entire B cell compartment has been inadequately studied to date. One patient with SLE and thrombocytopenia treated with rituximab showed blood B cell depletion and when splenectomy

was needed for inadequate response, splenic B cell depletion could also be demonstrated by pathology [100].

The main mechanism of B cell depletion by rituximab is still under study. Given the importance of the FcyRIIIa receptor phenotype on phagocytic cells in determining response to rituximab, B cells coated with rituximab may be cleared by antibody-dependent cell-mediated cytotoxicity as one proposed mechanism. Others include the induction of apoptosis by cross-linking of CD20 on the B cell surface, and the induction of complement-dependent cytotoxicity [101]. The latter mechanism, if vital, may point out subsets of lupus patients who are less likely to respond well to rituximab. Patients with genetic deficiency of complement (e.g. C1q or C4, who are already at higher risk of SLE) and those with acquired complement deficiency as part of high SLE disease activity have low complement levels and therefore a poor response to a complement-requiring pathway. In an animal model, C1q-deficient mice showed impaired rituximab responses to human CD-20 expressing lymphoma cells [102]. It is unlikely that mere B cell depletion accounts for the full clinical effect of rituximab, as B lymphocytes numbers plummet immediately after rituximab infusion, while clinical responses can take weeks to months.

A resetting of B cell homeostasis may be part of the clinical response to B cell depletion. Those patients with incomplete B cell depletion after rituximab have a mixture of naïve, memory, and pregerminal center CD38+IgD+ B cells, whereas successful B cell depletion leaves only memory B cells in the circulation [103]. Later recovery of B cell counts is associated with a return to B cell homeostasis, including resolution of B lymphopenia, the presence mainly of naïve B cells, and expansion of CD27-IgD- cells. Another group has argued, however, that the CD38+ population seen in active lupus patients mainly represents repopulating naïve B cells expressing higher levels of CD38, and a smaller population of pregerminal center cells, as seen in umbilical cord blood B cells or bone marrow transplants [99].

Rituximab in SLE Treatment

Small, largely uncontrolled, trials have now appeared for the use of rituximab in lupus nephritis. Looney et al. [104] treated 17 lupus patients, 7 of whom had proliferative glomerulonephritis. Three different doses of rituximab were used along with moderate-dose corticosteroids. The lowest dose of rituximab resulted in incomplete B cell depletion and the least clinical benefit. The highest dose rituximab was equivalent to that used in non-Hodgkin's lymphomas and resulted in improved clinical status in 6 of 8 patients. The level of anti-dsDNA antibody was not a reliable predictor of response to treatment overall, but its reduction did correlate well in 4 patients with high pretreatment levels and effective B cell depletion. Anti-chimeric antibodies (i.e. antibodies formed against the murine sequence in the rituximab antibody) were found in 6 of the 17 patients. These antibodies were seen more frequently in patients receiving lower-dose rituximab and in those with poor clinical responses.

A second open-label study of rituximab in SLE [105] included 6 lupus patients, 3 of whom had proliferative glomerulonephritis. The regimen used was borrowed from previous experience in treatment of rheumatoid arthritis and consisted of two 500 mg rituximab doses, two 750 mg cyclophosphamide infusions, and corticosteroids over two weeks. The results showed clinical benefit in all six, especially in fatigue, arthralgia, arthritis, and serositis. In two patients the benefits were long-lasting (2 and 3 years), and the other four relapsed at or after B cell repopulation. Levels of anti-dsDNA antibodies decreased in 2 of 3 patients with renal disease but varied in the group overall. This group has subsequently modified this regimen to include a

higher dose of rituximab at 1000 mg for each of two doses, and 13 of the next 15 patients (presenting with severe nephritis and/or cytopenias) have received this higher dose [106]. Overall, 9 of 21 patients responded well enough to require only one cycle of rituximab (follow-up 12 to 46 months), 6 have been re-treated, and 1 never depleted B cells and therefore did not respond. This group has also reported that rituximab treatment resulted in a reduction in anti-dsDNA and anti-histone antibodies while anti-Sm antibodies actually increased, correlating with higher BLyS levels in these patients [107]. Whether some autoantibody clones remain resistant to rituximab or unusually responsive to BLyS remains to be determined.

A recent open-label study of rituximab included a more homogeneous group of ten SLE patients with class III or IV proliferative glomerulonephritis [108]. The end points of the study were a complete remission of nephritis (normal serum creatinine and albumin, inactive urine sediment, 24 hour urine protein <500 mg) or partial remission (>50% improvement in renal parameters that were abnormal at baseline). Partial remissions were achieved rapidly (mean of 2 months) in 8 of 10 patients, and complete remission in 5 patients (mean of 3 months). These favorable responses are more rapid than is usually seen with the standard regimen of cyclophosphamide and corticosteroids. Levels of IgM plus IgG anti-dsDNA antibodies decreased in all patients, regardless of response, yet it has been pointed out that only a subset of the IgG antibodies may be pathogenic to the kidneys [109]. However, effective B cell depletion and clinical response correlated with a decrease in T helper cell activation as assessed by a decrease in CD40 ligand, CD69, and HLA-DR on CD4+ T cells. The 2 patients with less robust responses had shorter periods of B cell depletion. However, long-term B cell level does not correlate with clinical response, as 4 of 5 patients achieving remission had no deterioration even after B cell regeneration. Overall, rituximab was felt to act not only on the level of reducing B cell antibody production, but also by dampening T helper cell responses. In the future, it needs to be studied whether the activation state of T helper cells can be used as a biomarker for successful therapy of SLE.

Rituximab Side Effects

Side effects of rituximab are well-understood from the more than 300,000 NHL patients previously treated. Hypersensitivity reactions during infusion can include rash and fever and may necessitate stopping the drug. The risk of infection is theoretically increased since B- and T-lymphocyte responses are blunted by rituximab. When this occurs on the background of autoimmune diseases such as lupus, where lymphocyte dysfunction and complement deficiency can further decrease anti-microbial resistance, episodes of infection are expected to be more frequent than in a healthy population. However, antibody levels are generally maintained during rituximab treatment and infectious complications have not been increased in lymphoma trials.

The need for cotreatment along with rituximab remains to be explored in lupus. In RA, there were significantly better responses to use of cyclophosphamide plus rituximab rather than rituximab alone [110]. A further alternative might be the use of methotrexate with rituximab, which also produced improved RA clinical responses. In clinical experience with another biologic agent, infliximab, the addition of even low doses of methotrexate has been shown to decrease anti-chimeric antibody formation, and therefore avoids a loss of response that can be seen from in vivo neutralization of the therapeutic agent. Trials in RA have demonstrated that 1. a higher dose of rituximab (total dose $\geq$ 600 mg/m2) is more likely to produce a substantial clinical response (ACR 50 or better), 2. Clinical relapse occurs with a rise in autoantibodies, not just repopulation of the B lymphocyte compartment and 3. Seronegative RA patients do not respond

[111]. Beyond a direct effect on the pharmacokinetics of rituximab, cotreatment may have as yet undefined influences on inflammatory cell survival and distribution that account for its important clinical benefit when given along with rituximab.

Rituximab: Conclusions:

Despite treatment with full-dose rituximab, even when combined with corticosteroids, cyclophosphamide, or methotrexate, most lupus patients will eventually relapse. It remains to be studied if different dosages will improve responses, or if repeated or ongoing treatments to suppress rather than to cure the disease (as is done in the treatment of RA with TNFα modulators) will prove to be a viable long-term therapeutic option. The long-term risks of such an approach are currently unknown. Rituximab treatment has been used to date mainly in patients who have failed multiple previous therapies, which may indicate that therapeutic responses may be easier to achieve in the general population of lupus patients. Overall, the use of rituximab has the benefits of a large safety database from the experience in lymphoma patients, a plausible biologic explanation of its mode of action in targeting B cells alone, and growing utility in preliminary reports of studies in multiple autoimmune diseases.

Anti BLyS (BAFF, TALL-1, THANK, zTNF4)

BLyS is a member of the TNFα family and is an important survival factor for all B-lineage cells after they exit the bone marrow. BLyS expression is restricted to myeloid lineage cells (monocytes, macrophages, dendritic cells, and neutrophils) and is cleaved at the cell surface to release a biologically active, circulating molecule. BLyS has 3 known receptors, BAFFR, TACI, and BCMA, of which the first two appear most important for B cells. Signaling through the BLyS receptors activates the NF-κB pathways and upregulates cell survival signals such as BCL-2 and BCL-X_L (reviewed by [112]). Mouse models have demonstrated that transgenic overexpression of BLyS leads to autoantibody production and renal disease, that the BLyS level is elevated in mouse models of SLE, and that treatment with fusion proteins of BLyS receptors (TACI-Ig or BAFFR-Ig) decrease SLE progression and improves survival [113].

In human SLE, BLyS levels correlate with anti-dsDNA autoantibody production and with clinical disease activity when large populations are followed. In a study of 68 SLE patients followed for one year, BLyS levels were persistently elevated in 25% and intermittently up in a further 25%, with these estimates likely at the lower end of the actual range since high dose steroid treatment reduces BLyS levels [114]. Circulating BLyS levels were not useful in assessing SLE disease activity in individual patients, only in a pooled group of patients.

Treatments for SLE (and other autoimmune diseases such as rheumatoid arthritis) have been initiated using 3 products targeting BLyS: 1. LymphoStat-B (Human Genome Sciences, a human anti-BLyS monoclonal antibody; 2. TACI-Ig (ZymoGenetics/Serono), a BLyS receptor-immunoglobulin fusion protein that binds BLyS; and 3. BAFFR-Ig (Biogen/Genentech), a further BLyS receptor-immunoglobulin fusion protein. LymphoStat-B has progressed furthest in clinical trials. A phase I multicenter double-blind trial of 70 SLE patients used one or two infusions of drug compared to placebo. There was no excess of adverse events, and testing of circulating blood mononuclear cells confirmed a decrease in B lymphocytes numbers after LymphoStat-B. A phase II trial is nearing completion and results are scheduled for release in the fall of 2005. On April 6, 2005, preliminary results of a phase II trial of LymphoStat-B in rheumatoid arthritis were released. Here, 283 patients received placebo or one of 3 doses of LymphoStat-B (1mg/kg, 4 mg/kg, 10 mg/kg) at weeks 0, 2, 4, then every 4 weeks. After 24

weeks, the primary outcome measure, an ACR20 response, was met only in the lowest dose group (36% vs. 17% for placebo, p=0.011). Numbers of circulating B cells (CD20+ and other subsets) and levels of rheumatoid factor dropped significantly in all actively-treated groups. Details on which B cell subsets were reduced and to what extent are not yet available.

Anti-CD22

CD22 is a B lymphocyte-restricted sialoglycoprotein expressed only on resting and activated mature B cells [115]. Unlike CD20, after ligand binding, CD22 is rapidly internalized, causing costimulation of normal cells and apoptosis of lymphoma cells [116]. Mice deficient in CD22 have B cells with reduced life span and increased apoptosis, giving some similarities in function for CD22 and BLyS [117]. The precise function of CD22 remains under study, with suggested roles in B cell signal transduction through the B cell antigen receptor, and as an adhesion molecule [116, 118].

A mouse monoclonal antibody to CD22 has been humanized to make an IgG1 antibody called epratuzumab. Over 300 patients with B cell lymphomas have now been treated in initial studies. In phase I/II studies of aggressive, mostly relapsed non-Hodgkin's lymphoma, the drug has been given once weekly for four weeks in a regimen similar to that used for rituximab [119]. The mean serum half life was 23.9 days, and tumor mass reduction was noted in 20% of the 56 patients.

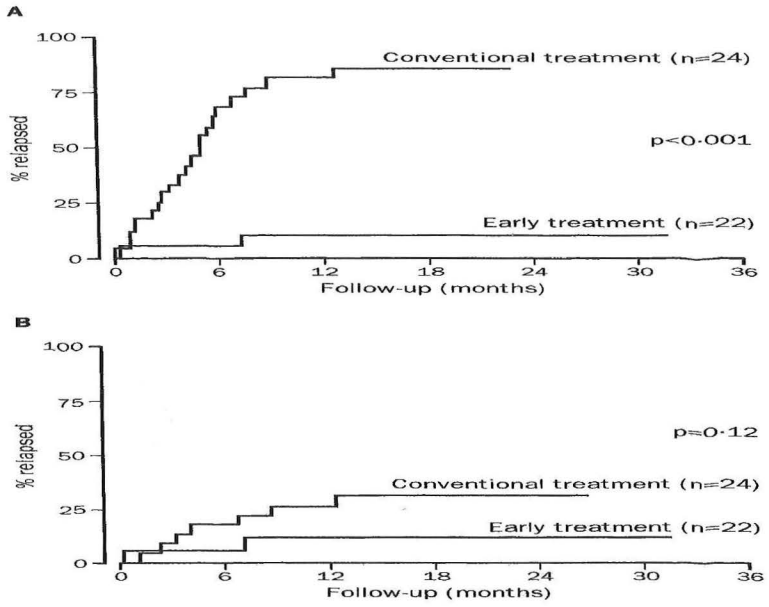
In SLE, on open trial of 14 patients showed initial promise [120]. Patients had a median 8 years duration of SLE, at least 1 elevated autoantibody, and moderately active current disease activity (BILAG 6-12). The infusion of epratuzumab was generally rapidly completed in half an hour and was given every 2 weeks for four doses. Three adverse events have been noted: sleepiness, herpes zoster, and otitis media. The BILAG global disease activity decreased in 11/11 patients immediately after treatment (by 50% or more in 9 of 14 patients) and also at week 10 (11/11 patients) and week 18 (7 of 9 patients). B lymphocyte levels decreased immediately after therapy and remained lowered at week 4. There was no consistent effect on T cell levels, immunoglobulins, and autoantibodies. Such limited results form the basis for Phase III trials in SLE in the near future. Epratuzumab is also going into phase I/II trials for Sjogren's syndrome, Waldenstrom's macroglobulinemia, and childhood ALL

At UT Southwestern, Dr. Ellen Vitetta has produced a novel anti-CD22 antibody coupled to immunotoxin (ricin). This agent has the advantage of killing its target cell, regardless of Fc receptor phenotype. There have now been successful clinical trials for lymphoma, and we have recently begun the first study in an autoimmune disease, ITP.

Targeting anti-dsDNA Antibodies in Therapy

Although there is controversy regarding the significance of anti-dsDNA antibody titers in predicting a flare of SLE, there is evidence that rising antibody titers correlate with a 2 to 3 fold increase in risk of a flare within 4 months [121]. The clinical features most likely to worsen with a rise of anti-dsDNA antibodies are glomerulonephritis and vasculitis [122]. Efforts have been made to prevent flares in response to rising anti-dsDNA antibody titers. Bootsma et al. used a strategy of adding prednisolone 30 mg/d (and tapering to baseline over 18 weeks) to the ongoing treatment of SLE patients with at least a 25% rise in anti-dsDNA titers [123]. During a 3 year study, 46 of 156 SLE patients had a rise in anti-dsDNA titers, with 22 assigned to receive the prednisolone regimen and 24 remaining on conventional treatment. Relapses in SLE disease activity occurred in 20 conventionally-treated patients, versus 2 in the prednisolone group.

Despite the use of extra prednisolone in stable patients, the prevention of flares resulted in no differences in cumulative steroid doses. Members of the same group have since studied the prophylactic use of mycophenolate mofetil (Cellcept) in a cohort of SLE patients [124]. 10 of 36 patients had rising anti-dsDNA titers during the study and received 2 grams/day mycophenolate mofetil for 6 months. None of the 10 patients relapsed on this regimen, and all had falling titers of anti-dsDNA antibodies. It must be pointed out that the prednisolone study had a particularly strong correlation between rising serologic titers and clinical flare (20 of 22 conventionally-treated patients flared), and that no control group was included in the mycophenolate mofetil study. Other researchers describe a much weaker association between worsening serology and clinical flares of SLE [125, 126]. However, if an individual patient's pattern of laboratory and clinical abnormalities is similar with each disease flare, prophylactic treatment can be justified based on these results.



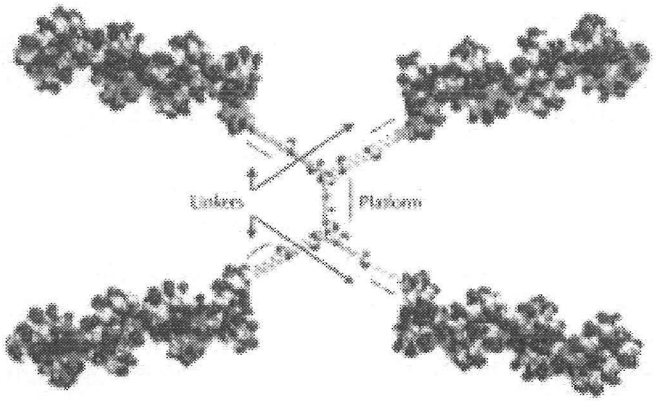
Corticosteroids: All relapses

Corticosteroids: Major relapses

LJP 394: targeting anti-dsDNA as therapy of lupus nephritis

LJP 394 (abetimus sodium, Riquent) is a rationally-designed agent to bind anti-dsDNA antibodies in the circulation and on cells. It consists of four deoxynucleotide sequences tethered to a triethylene glycol backbone. Although this agent may aid in the binding and clearance of circulating autoantibodies, the more important proposed mechanism of action may be the induction of anergy or apoptosis by binding to the surface of B lymphocytes, leading to antigen-specific tolerance [127].

LJP 394 has been extensively studied in 13 clinical trials involving 873 SLE patients. The phase 2/3 randomized controlled trial of 230 patients enrolled SLE patients with a history of nephritis and assessed for a renal flare (increased 24 hr proteinuria, serum creatinine, or hematuria; and decreased 24 hr creatinine clearance), the time to a major SLE flare (initiation or increased dose of corticosteroids or cyclophosphamide, hospitalization or death from SLE), and



LJP 394

the time to requirement of high dose corticosteroids or cyclophosphamide [128]. In an intent to treat analysis, the trial showed no significant differences in time to and incidence of renal flares among treatment groups, but LJP 394 recipients had a longer time to institution of high dose steroids or cyclophosphamide (p=0.03) and required fewer courses of such treatments (p=0.026). A retrospective analysis of the 90% of trial subjects who had high affinity anti-dsDNA antibodies showed that the time to and incidence of renal and SLE flares were statistically different in this population compared to placebo. These results led to a prospective definition of the high affinity anti-dsDNA antibody group for the subsequent phase 3 trial.

The phase 3 trial of LJP 394 included 145 patients on study drug, 153 on placebo [129]. When data from LJP 394 and placebo patients were pooled, the premise behind this treatment approach was confirmed by demonstrating that decreases in anti-dsDNA (of about 25%) were associated with a lower risk of renal flare. In the study, there was a trend towards fewer renal flares in LJP 394-treated patients (12% LJP 394 vs. 16% placebo) and fewer major SLE flares (24% LJP 394 vs. 31% placebo), but these did not achieve statistical significance. There was a longer median time to renal flare in the LJP 394 group (123 months vs. 89 months placebo) and to major SLE flare (55 months vs. 42 months placebo). There was no difference in the requirements for corticosteroids or cyclophosphamide between the two groups. The LJP 394 patients had similar numbers of serious adverse events as the placebo subjects during treatment for up to 22 months. Given the lack of statistical significance to the phase 3 results, the FDA did not approve LJP 394 in an October 2004 decision. La Jolla Pharmaceutical Company has begun an even larger clinical trial to demonstrate clinical efficacy, but at a cost to the company of $2.5 million a month, there are doubts whether the study can be completed. The company is lobbying to perform a post-marketing trial, instead.

Conclusions

Over 100 autoantibodies are now recognized in SLE. Although the initial inciting antigens that trigger the clinical disease in SLE are not known, studies have demonstrated cross-reactivity between nuclear antigens and some viral sequences. Autoantibodies can be found for 10 or more years in the serum of patients who later develop clinical SLE, with an accumulation of new antibody specificities that peaks near the time of clinical disease outbreak. The antibody response to many cellular antigens shows evidence of isotype switching and affinity maturation by B cells, and epitope spreading over time. Current therapies for SLE rely on general immune suppression and nonspecific reduction of inflammatory cells. New therapies target B lymphocytes by outright killing or by immune modulation, moving the focus of SLE treatment to an attack on the humoral arm of the immune system.

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