# CHAPTER 3

# REVIEW LITERATURE

## Systemic Lupus Erythematosus

Systemic lupus erythematosus (SLE) is an autoimmune rheumatic disease characterized by widespread inflammation affecting virtually every organ or system in the body and the production of multiple autoantibodies, typically antinuclear and anti-DNA antibodies. In some patients, autoantibodies are also produced against platelets, lymphocytes, and other cellular antigens. It has a multifactorial etiology and affects mainly women during the childbearing years. Contributory factors include hormonal, genetic, and environmental components. As in any autoimmune condition, it is believed that the presence of susceptibility genes in a predisposed individual can be triggered by an initial stimulus (probably environmental) which enables a certain critical threshold to be attained, leading to the development of clinical features. Polyclonal B cell activation and excessive T cell help are involved in the pathway that incorporates the perturbation of the immune response.

Much work has been done in the last 30 years to dissect and understand the complex array of immunological disturbances, which culminate in the diverse clinical features, which characterize the disease. A variety of murine and other animal models of lupus exist, which have been used extensively to investigate the immunogenetic and cellular abnormalities, which are characteristic of human SLE.

## Epidemiology and natural history

Lupus is found worldwide. The prevalence of the disease depends on ethnic background. The highest prevalence is seen in Asians and Blacks (23, 24). As in other systemic autoimmune diseases, there is a striking preponderance in women, especially during childbearing age. This preponderance is related to hormonal status. Animal studies have shown that estrogens have a facilitating effect on disease expression, whereas androgens have a suppressive effect. The importance of estrogens is further substantiated by the fact that changes in the hormonal homeostasis (*e*.g., at onset of puberty, during use of oral anti-contraceptives, and during pregnancy and puerperium) are associated with an increased frequency of lupus onset and disease flare up. The genetic susceptibility is illustrated by the concordance of the disease in twins, occurrence of familial aggregation, and association with certain genes, mainly human leukocyte antigens (HLA). The major epidemiologic characteristics of systemic lupus erythematosus are shown in table 1 (19).

The marked improvement in survival during the past 20 years is probably the consequence of greater awareness of the condition, increased availability of autoantibody testing to make the diagnosis earlier, the more judicious use of steroids and other immunosuppressive drugs, and undoubtedly the widespread availability of dialysis and renal transplantation. It does, however, remain a disease with the potential to cause considerable morbidity and increased mortality in a subset of patients.

## Classification of SLE

The American Rheumatism Association (now the American College of Rheumatology, ACR) initially published classification criteria in 1971 (25), which were revised in 1982(26). A further revision has recently been proposed(27). The criteria are for the classification of the disease rather than for use as a diagnostic tool, although in practice there is a blurring of this distinction. The 1997 updated criteria

(28) are outlined in Table 2. For the purpose of identifying patients in clinical studies, it is determined that a patient has SLE when at least four of these criteria are present, serially or simultaneously, during any interval of observation(27).

**Table 1:** Epidemiologic and genetic characteristics of systemic lupus erythematosus

| Epidemiology | Genetics |
|---|---|
| Prevalence: between 25 and 250 per 100,000 persons, depending on racial and geographic background | Concordancy in twins<br>Monozygotic: 50–60%<br>Dizygotic: 5–10% |
| Race: more prevalent in Asians and blacks | Familial aggregation in 10% |
| Gender: female preponderance; gender ratio between 20 and 40 years; male:female, 1:9 | Association with the following:<br>HLA: B7, B8, DR2, DR3,$DQ_{w}1$ |
| Age: onset mainly between 20–40 years | Complement:<br>C4A Q0<br>C1q or C4 deficiency<br>Fc receptor IIA low-affinity phenotype<br>X chromosome ? |

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**Table 2**: Revised American Rheumatism Association criteria for classification of systemic lupus erythematosus(27).

1 Malar rash

2 Discoid rash

3 Photosensitivity

4 Oral ulcers

5 Arthritis

6 Serositis

- a. pleuritis, or
- b. pericarditis

7 Renal disorder

- a. proteinuria > 0.5g/24h or 3+, persistently, or
- b. cellular casts

8 Neurological disorder

- a. seizures or
- b. psychosis (having excluded other causes)

9 Haematological disorder

- a. haemolytic anaemia or
- b. leucopaenia or <4.0 x$10^{9}$/1 on two or more occasions
- c. lymphopaenia or <1.5 x$10^{9}$/1 on two or more occasions
- d. thrombocytopaenia <100 x$10^{9}$/1

10 Immunological disorders

- a. raised anti-native DNA antibody binding or
- b. anti-Sm antibody or
- c. positive finding of antiphospholipid antibodies based on
    - i. an abnormal serum level of IgG or IgM anticardiolipin antibodies
    - ii. A positive test result for lupus anticoagulant using a standard method
    - iii. A false–positive serological test for syphilis, present for at least 6 months

11 Anti-nuclear antibody in raised titer

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## Clinical symptoms

SLE is characterized by a plethora of clinical features. Non-specific features such as severe fatigue, fever, anorexia, weight loss and lymphadenopathy are common and can form a major part of many patients' illness.

The clinical expression of SLE is extremely varied, but the most common findings include: fever, erythematosus rash, arthritis, serositis (pleuritis, pericarditis), and nephritis. Skin photosensitivity, oral ulcers, hematologic disorders, and neurological abnormalities are also seen. In most published series until very recently, renal disease has been the most common cause of death in patients with lupus and remains a cause of considerable morbidity in up to 30% of patients. The World Health Organization (WHO) has subdivided renal lupus into five major categories based on renal biopsy results. In addition, end-stage renal disease with completely sclerosed glomeruli, tubular interstitial disease and the overlap of lupus nephritis and multiple small thrombi associated with antiphospholipid antibodies, can also occur. The cumulative incidence of clinical symptoms in Systemic Lupus Erythematosus is shown in table 3.

**Table 3:** Cumulative incidence of clinical symptoms in systemic lupus erythematosus

| | Percent |
|---|---|
| **Frequency of major clinical symptoms** | |
| Musculoarticular symptoms | 60–95 |
| Cutaneous manifestations | 55-80 |
| Renal involvement | 40–55 |
| Neuropsychiatric disease | 30–60 |
| Pulmonary and cardiac disease | 20–40 |
| Hematologic abnormalities | 60–85 |

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# Laboratory tests in diagnosis and monitoring of SLE

Laboratories, and particularly clinical immunology laboratories, play an essential role in diagnosis and monitoring of systemic lupus erythematosus (SLE). Many tests are done on patients with suspected lupus in order to establish a diagnosis. In patients with SLE, tests are performed to determine which organs are involved, and also to determine the degree of activity of lupus i.e., antinuclear antibody tests, complement, complete blood count (CBC), kidney function tests, brain involvement etc. In this regard, we focus on autoantibodies and complement in SLE.

## Autoantibodies

In almost all patients, antibodies are formed against nuclear antigens, as detected by antinuclear antibody (ANA) testing. The overview of autoantibody formation in Systemic Lupus Erythematosus is shown in table 4.

### Anti-nuclear antibodies

More than 95% of patients with SLE have a positive antinuclear antibody, detected by indirect immunofluorescence usually on nuclei from cell lines such as HEp-2 (human epithelial cell). Different staining patterns correlate with different antibody binding, such as the homogeneous pattern corresponding to the binding to double-stranded DNA and/or histones. Speckled or nucleolar patterns can also occur. ANA-negative patients generally have antibodies to Ro and/or La and tend to have less renal disease(28).

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### Anti-DNA antibodies

Antibodies to dsDNA are found in between 40 and 90% of patients with SLE. Many studies have shown an association between anti-dsDNA antibodies, particularly IgG antibodies, and the occurrence and severity of renal disease(29-32). They have

also been shown to correlate with cardiopulmonary disease and global activity score as assessed by the BILAG index (33). Anti-dsDNA antibodies have been eluted from kidneys in murine models of SLE and antibodies showing anti-nuclear activity that could be partially inhibited by dsDNA have been eluted from the kidneys of patients that have died from renal lupus(34).

**Antinucleosome antibodies**

Anti-nucleosome antibodies, which are responsible for the "so-called" LE phenomenon, play an important role in the pathogenesis of SLE(7, 21, 35). Recently, a growing interest on antinucleosome antibodies as a diagnostic tool has emerged in the literature(15, 22, 36). Antinucleosome antibodies occur up to 80 % of SLE patients(15, 22, 36). Conflicting results have however been reported concerning the presence of antinucleosome anti-bodies in other connective tissue diseases(15, 22, 36) and their correlation, in lupus patients, with disease activity and renal involvement(15, 22).

**Antibodies to extractable nuclear antigens**

Antibodies to the extractable nuclear antigens (Ro, La, Sm, RNP, etc.) can be found in up to one-third of patients. Antibodies to La are particularly associated with the coincidence of Sjögren's syndrome and SLE. Anti-Ro antibodies are associated with photosensitivity, the sub-acute cutaneous form of lupus and possibly with glomerular inflammation, and both antigens are associated with the neonatal lupus syndrome. Antibodies to RNP are associated with undifferentiated autoimmune rheumatic disease, and anti-Sm antibodies, while being virtually diagnostic of SLE, are not associated with any particular disease feature, although they are found with an increased frequency in black lupus patients (30%) compared with Caucasians (5%).

**Anti-phospholipid antibodies (aPL)**

Anti-phospholipid antibodies are a heterogeneous group of autoantibodies that exhibit a broad range of target specificities, all recognizing various combination of phospholipids, phospholipid-binding proteins or both. Antiphospholipid antibodies

interfere with coagulation mechanisms resulting in a pro-thrombotic state. The most commonly detected subgroups of aPL are Lupus Anticoagulant (LAC), anti cardiolipin antibodies and anti-β2 glycoprotein I antibodies. Anti-phospholipid antibodies have been recently included into ACR criteria for SLE (37). The true target of aPL is represented by the complex phospholipid-β2 glycoprotein I or even by the sole β2 glycoprotein I. LAC and/or antiphospholipid antibodies are found in 30-40% of lupus patients. Anti phospholipid antibodies are also found in patients with the "so-called" primary anti-phospholipid syndrome. Higher titers of aPL antibodies are generally found in the phases of disease activity. However, serum titer of aPL antibodies is not particularly useful for monitoring disease activity (38).

**Table 4:** Cumulative incidence of autoantibody formation in systemic lupus erythematosus (24)

| | Percent |
|---|---|
| **Occurrence of major autoantibody specificities** | |
| Antinuclear autoantibody | 95 |
| Anti–double-stranded DNA | 60-75 |
| Antihistone | 50-70 |
| Antinucleosome | up to 80 |
| Anti-Sm | 10-30 |
| Anti-ribonucleoprotein (RNP) | 10-30 |
| Anti–Sjögren's syndrome (SS-A) (Ro) | 20-60 |
| Anti-SS-B (La) | 15-40 |
| Anticardiolipin | 10-30 |
| Antierythrocyte | 50-60 |
| Antilymphocyte | 50-70 |
| Antithrombocyte | 10-30 |

## Complement

Assays of complement levels in serum are one of the standard assays used to assist the clinical management of patients with SLE(39). The majority of laboratories measure antigenic concentrations of C3 and C4. A smaller number of laboratories also routinely provide a functional measurement of the activity of the whole complement pathway from classical pathway activation through to formation of the membrane attack complex, such as the CH50 (complement haemolysis 50%) test.

The dominant pathway for complement activation in SLE is the classical pathway, triggered by the interaction of C1q with immune complexes. Classical pathway complement protein levels are reduced in association with active disease, especially C1, C4 and C2 levels. Levels of C3 are typically at the lower end of the normal range and only occasionally severely depressed. Levels of C3 are maintained because of the regulatory mechanisms that control classical pathway complement activation *in vivo*, especially the activity of C4 binding protein that inhibits classical pathway activation(40). When C3 levels are reduced, this is usually associated with reduced levels of factor B, indicating amplification of C3 turnover *in vivo* by the amplification loop of the alternative pathway(41, 42).

In patients with established disease, regular measurement of complement activity is a helpful guide to disease activity(30). At the onset of disease, complement measurements may have diagnostic value. Evidence of complement activation is a marker of the family of diseases in which immune-complex-mediated pathology is prominent. Although a rare finding, it is also important to consider whether a patient might have an inherited complement deficiency underlying the disease. Assay of C4 and C3 levels alone may not alert the practitioner to the presence of complement deficiency. Indeed in the case of C1 deficiency, C4 and C3 levels may be high because of reduced consumption of classical pathway proteins(43).

## Autoimmunity in SLE

The precise immune disturbances leading to the development of SLE remain unknown, although various clues have been provided by studies of animal models(44). Current research involves attempts to understand the process of production of pathogenic autoantibodies and the roles played by cytokines, adhesion molecules and apoptosis.

Formation of antinuclear autoantibodies, especially against double-stranded (ds) DNA, is a hallmark of systemic lupus erythematosus (SLE). It is generally assumed that these anti-dsDNA antibodies associate in the development of lesions in SLE. Until recently, it was rather curious why these multi-molecular complexes, normally hidden in the nucleus, become immunogenic and targets for autoimmune responses. Especially, in view of the fact that naked dsDNA is hardly immunogenic(2, 3), and most likely in this form can not be considered as a major autoantigen in SLE. The primary event inducing the formation of anti-dsDNA antibodies has always been puzzling, since it has been very difficult to demonstrate the presence of free DNA in serum of SLE patients, but when DNA was found it occurred in the form of (oligo) nucleosomes(1). A potential source for these nucleosomes are apoptotic cells, since nucleosomes were spontaneously released from cultured normal murine spleen cells, whereas this process did not occur when apoptosis was blocked with zinc sulfate(45). Also during culture of human lymphocytes nucleosome release occurred, which was strongly correlated with the degree of lymphocyte apoptosis(46).

In the last decade several pieces of evidence have shed new light on the mechanisms leading to autoimmune responses and the pathogenesis of SLE(7, 47). Lupus nephritis is one of the most serious complications in SLE, occurring in up to 55% of patients with SLE. Traditionally, it was thought that lupus nephritis resulted from the glomerular deposition of DNA/anti-DNA complexes. However, DNA/anti-DNA complexes are hardly nephritogenic(48). Also with regard to the pathogenesis of lupus nephritis several observations have provided new clues for the events that lead to glomerular inflammation in SLE.

In 1995 Berden et al presented a new hypothesis for the development of lupus nephritis (49) shown in Figure 1. Disturbed apoptosis (either too much, at the wrong place or delayed) leads to the persistence of autoreactive T and B cells. If the rate of apoptosis overflows the phagocytic removal of apoptotic cells or phagocytosis is impaired, apoptotic cell derived material will be released. This includes nucleosomes and other nuclear autoantigens. During apoptosis these autoantigens can be modified, which can make them more immunogenic. This (increased) exposure of (modified) nucleosomes to the immune system leads to the formation of autoantibodies to nucleosomes and dsDNA.

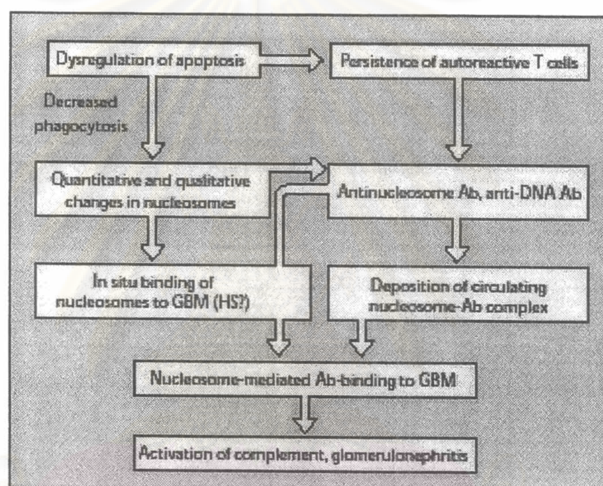


**Figure 1:** Hypothesis for the immune dysregulation in SLE and the development of lupus nephritis (49).

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## The nucleosome

Nucleosomes are the basic units of chromatin. In eukaryotic cell nucleus, 2 m of DNA are densely packed in the form of chromatin. In chromatin, adjacent nucleosomes are linked by about 60 bp of DNA (Figure 2)(3). Each nucleosome consists of pairs of the histone peptides H2A, H2B, H3 and H4, forming the histone-octamer. Around this octamer 146 bp of DNA are wrapped in two superhelical turns. At the outside of this core particle, a molecule of H1 is located at the point where DNA enters and exits the particle. The amino-terminal portions of the histones carry many positive charges, in contrast to the DNA which part is anionic. Through this combination of cationic and anionic regions the overall charge of the nucleosome is neutral with a pI of 6.9(47).

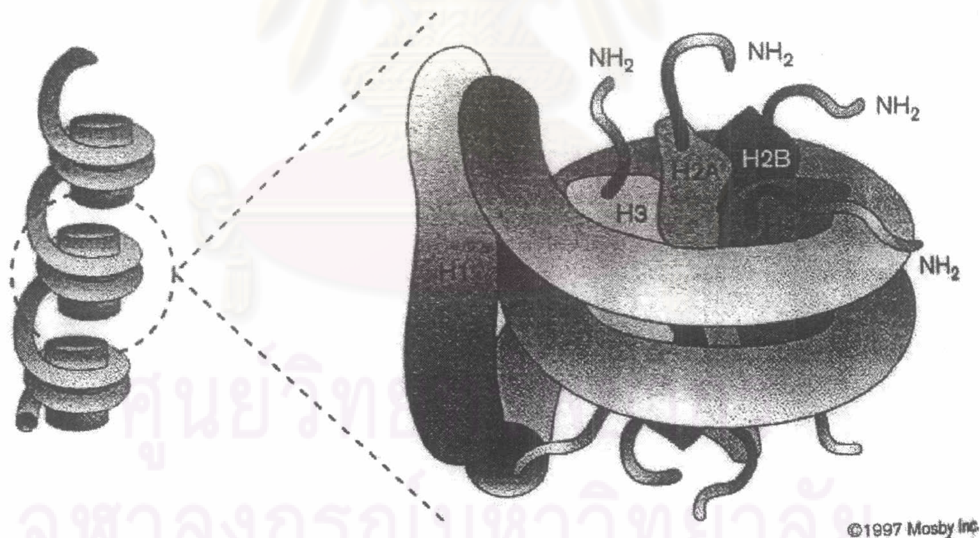


**Figure 2**: Chromatin (left part) is a polymer structure of nucleosomes connected by protein-free DNA. The nucleosome (right part) consists of a histone-octamer comprising pairs of the histone peptides H2A, H2B, H3 and H4, around which dsDNA is wrapped twice. H1 is located at the outside where DNA enters and exits the core particle.

## Apoptosis in SLE

The dysfunction of programmed cell death (apoptosis) has been found to be part of numerous human diseases, particularly the autoimmune diseases(50).

As a consequence of apoptosis, nuclear components are brought to the surface of the dying cells within "blebs" and become exposed to the immune system. Casciola-Rosen et al. found that in apoptotic keratinocytes many nuclear and cytoplasmic antigens, that are characteristic targets for autoantibodies, are clustered in high concentrations in the cell membrane(51). Furthermore, alterations of cellular components during apoptotic degradation, occurring via posttranslational modifications such as phosphorylation, oxidation, or citrullination, could induce immunogenicity (10, 52).

The first finding that apoptosis was associated with lupus came from the discovery that MRL/*lpr* lupus mice had a functional Fas deficiency. Binding of the Fas ligand to the Fas receptor (CD95), present on activated T and B cells, leads to apoptosis. The deficiency of the Fas ligand like in *gld* mice, also leads to the same lupus phenotype as in MRL/*lpr* mice(53). The defects in Fas and Fas ligand, respectively, result in an incomplete elimination of peripheral autoreactive cells as this elimination occurs predominantly by Fas mediated apoptosis (54). Another example of inhibition of apoptosis leading to autoimmunity is transgenic overexpression of Bcl-2. When the Bcl-2 gene was put under the control of an immunoglobulin promoter, the number of autoreactive B cells increased, leading to the production of anti-nucleosome autoantibodies and glomerulonephritis(55).

According to the demonstration that the autoimmune lymphoproliferative syndrome in the *lpr* and *gld* mouse strains was based on a single gene defect, studies in human were performed to detect similar functional defects in Fas or Fas ligand. In patients with SLE, Fas-related defects in apoptosis are less clear(56). Patients with a Fas or Fas ligand deficiency develop an autoimmune lymphoproliferative syndrome (ALPS)(57). Only a quarter of the patients develop antinuclear antibodies and

glomerulonephritis is rarely seen. In contrast to the animal models, in human SLE no clear-cut genetic defects in the Fas pathway have been detected so far. However, several studies have been performed in which soluble Fas was measured in SLE patients. These studies demonstrated elevated levels of soluble Fas in lupus patients(58, 59), increased Bcl-2 expression(60), increased levels of circulating apoptotic cells(61, 62) and increased "spontaneous" apoptosis in vitro(46).

Dysregulation of apoptosis can contribute to the pathogenesis of lupus nephritis in two different ways. First, apoptosis seems to be an important mechanism to induce tolerance of T cells towards self-antigens (63, 64). This indicates that persistence of autoreactive T cells may cause by a disturbance in apoptosis and in this way affect the magnitude of the anti-nucleosomal immune response. As mentioned above, one of the consequences of a defective Fas antigen is breakdown of peripheral T cell tolerance. Second, autoreactive lymphocytes that have escaped apoptosis may be "primed" for apoptosis (47). Some of these cells may be induced to become apoptosis by other mechanisms (unrelated to Fas). It is hard to draw a clear-cut conclusion. Although suggested by animal studies, since no genetically defined defects in the major, Fas-mediated, apoptotic pathway have been detected in human SLE. Therefore, a hereditary defect in the induction of apoptosis as a mechanism of elimination of autoreactive lymphocytes does not seem a prerequisite factor for the development of human autoimmune disorders.

However abnormal apoptosis in itself is not necessarily to be harmful if the removal of apoptotic cells is rapid and complete. Hence, impairment of apoptotic cells clearance is probably more important than disturbance in apoptosis itself.

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**Phagocytosis of apoptotic cells in SLE**

The interest in the body's cleaning machinery for apoptotic cells has increased after the finding that apoptotic cells are the source of autoantigens. A seminal observation of Rosen et al showed this for the first time. They found that autoantigens become clustered in surface blebs after induction of apoptosis of keratinocytes with UV light (51). The smaller blebs contain SS-A (52 kD), ribosomal P protein, α-fodrin and Jo-1, while the larger apoptotic bodies contain nucleosomes, SS-A (60 kD), SS-B, Sm, SnRNP complexes, poly (ADP-ribose) polymerase (PARP) and other autoantigens (65). Removal of apoptotic cells occurs very effectively via phagocytosis by bystander (semi-professional) or professional phagocytes like macrophages and monocytes (66). Rapid elimination of apoptotic cells is important as it prevents the release of (toxic) cell constituents like cytolytic enzymes. Sufficient removal of apoptotic cells therefore also seems important for the prevention of (excessive) autoantigenic exposure(67).

An important feature of apoptotic cell removal by macrophages is the induction of an anti-inflammatory reaction (68, 69) instead of release of pro-inflammatory cytokines as observed if phagocytosis occurs via Fc receptor (70).

Because all autoantigens targeted in SLE are either located in small or large apoptotic blebs or at the surface of apoptotic cells, Tax et al (49) and others (71) have postulated that a defective phagocytosis of apoptotic cells may be a pivotal feature in the generation of the autoimmunity. This impaired clearance by phagocytosis may lead to the release of nuclear antigens including nucleosomes (72), because the major pathway for generation of nucleosomes is apoptosis.

The indications that the removal of apoptotic cells and their antigenic structures is relevant in the pathogenesis of SLE are delivered by a recent study performed with Dnase1-deficient mice. Napirei et al. measured Dnase1 activity in the sera of SLE patients and found lower levels than in normal controls (73). Dnase1 might have a protective task in the removal of DNA from nucleoprotein complexes so

preventing immune stimulation. Indeed, deletion of Dnase1 resulted in the occurrence of classical symptoms of SLE, including the production of antinuclear antibodies and the development of glomerulonephritis.

Furthermore, when phagocytosis occurs via Fc or complement receptors, this will usually be accompanied by the production of pro-inflammatory cytokines, in contrast to the direct phagocytosis of apoptotic cells, which can actually have an anti-inflammatory and immunosuppressive effect, so contributing to the process of inflammation which is characteristic for autoimmune diseases (68).

## Immunogenicity of nucleosomes

Since naked dsDNA has long been regarded as the major autoantigen in SLE, many attempts have been made to immunize with dsDNA in all sort of forms and conditions. However these procedures failed to induce anti-dsDNA antibodies with lupus specific characteristics (67). The first positive result has been derived after immunization with dsDNA complexed to histone-like DNA binding proteins from either viral or protozoal origin. The antibodies formed were directed against dsDNA and nucleosomes (74). Seminal studies by Datta et al showed that in the SNF1 murine lupus model 50% of the pathogenic T helper cells were directed against nucleosomes. These Th cells did not only provide help for the production of nucleosome specific antibodies, but also for anti-dsDNA and anti-histone antibodies, a concept known as antigen spreading (figure 3) (5). Figure 4 shows the binding of T cell co-stimulatory molecules with their corresponding B cell receptors, which required for contact-dependent antibody production.

This finding shed new light on the initiation of the anti-dsDNA antibody response: not dsDNA but nucleosome is the driving autoantigen in SLE (49). Subsequently, similar observations have been reported in human SLE (15, 75). These nucleosome specific T cells respond to histone epitopes on MHC class II molecules presented after processing of nucleosomal material by APC (76, 77). However it has

been indicated that APC can present epitopes derived from engulfed apoptotic cells to T cells (78, 79). Surprisingly, in view of the exogenous source of the antigen, this antigen presentation took place via MHC class I molecules. So, these data demonstrated that T cells responds to nucleosomal epitopes are present in both human and lupus mice. In fact, in lupus mice nucleosome-specific CD4+ T cells are detected long before lupus mice produce pathogenic autoantibodies, suggesting that these cells play a role in triggering the disease (5).

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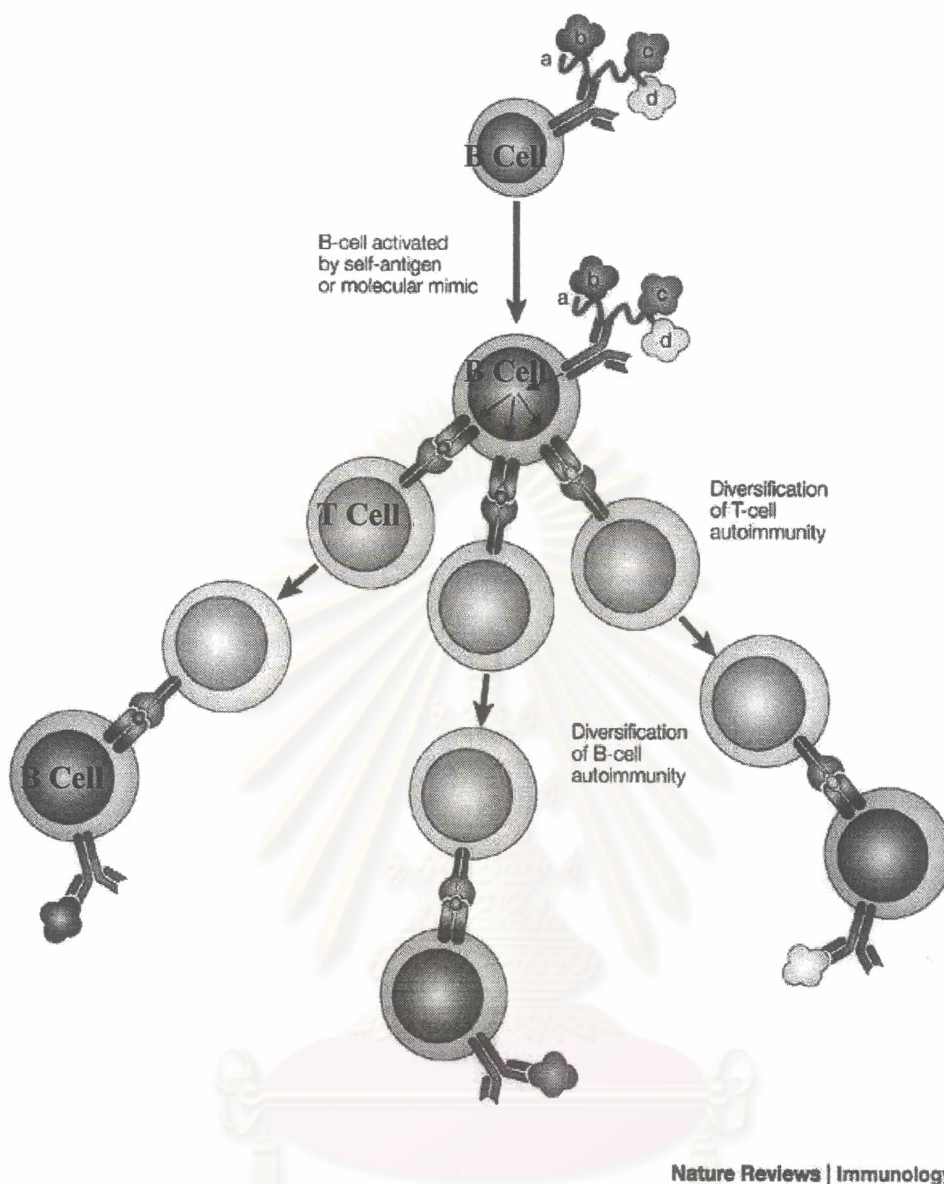


**Figure 3: Mechanism of epitope spreading.** A B cell, specific for determinant 'a', takes up through its B-cell receptor a multideterminant antigen that consists of multiple proteins or protein–nucleic acid complexes ('b'–'d'). These multiple T-cell-antigenic determinants, all arising from one initial complex antigen, are processed by the B cell and presented in the context of major histocompatiblily complex (MHC) class II. The single anti-a-specific B cell can thereby activate, and receive help from, a diverse set of T cells. These, in turn, can provide help to a diverse group of B cells that can recognize separate B-cell antigenic determinants on any part of the complex antigen, resulting in the synthesis of anti-b, c and d antibodies. (Nature © Macmillan Publishers Ltd 2001 Registered No. 785998 England)

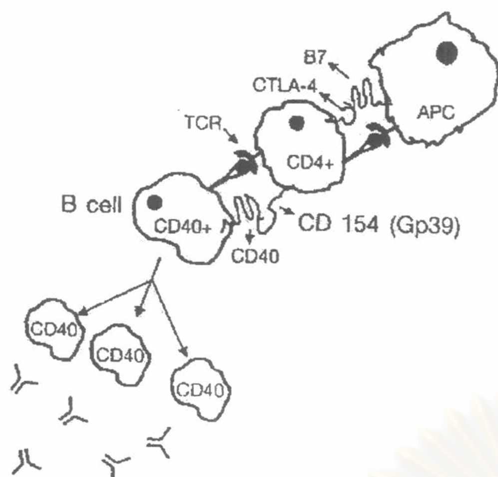


**Figure 4: Antigen-dependent antibody production.**
Antigens bound to B cells receptors or processed by antigen presenting cells (APC) are presented on the cell surface in conjunction with MHC class II molecules. CD4+ T cells recognizing MHC-antigen complexes are activated resulting in expression of gp39. Interaction between T cell gp39 and B cell CD40 results in B cell proliferation and antigen-specific antibody production.

Work toward identifying the T cell autoepitopes for nucleosomes has progressed: 3 major regions in core histones, one in H2B (residues 10-33), and two in H4, at positions 16-39 and 71-94, have been demonstrated to contain the peptide epitopes recognized by nucleosome-specific Th cell clones derived from nephritis (NZB X SWR)$F_1$ (SNF1) lupus mice (76). Two additional regions in H3, at position 88-102 and 118-132, also evoked strong proliferative responses of splenic CD4+ T cells from SNF1 mice (76). The importance of nucleosomes as immunogenic particles was underlined by the finding that injection of nucleosomal epitopes into preautoimmune mice could accelerate the development of severe glomerulonephritis (76).

An intriguing question is the nature of the epitopes recognized by the pathogenic Th cells. These nucleosome-specific Th clones were not activated by free DNA or histones, the components of nucleosomes. This result indicates that either nucleosomes are taken up more efficiently by antigen presenting cells, or that critical Th cell epitopes in the histones are protected from degradation by being bound to DNA (5). Another possibility is that after antigen processing of nucleosomes, MHC class II molecules present self-peptides that were previously cryptic. It has also been suggested that the presence of endoplasmic reticulum or nuclear membrane within the apoptotic cells can cause an increased generation of reactive oxygen species, which

may induce oxidative modification of autoantigens. The unique peptide fragments generated in this way could then, in genetically susceptible individuals, be presented to Th cells and induce an autoimmune response (51).

Interestingly these pathogenic nucleosome-specific Th cells were only present in lupus-prone mice and not detected in normal mice (5). In normal controls, apoptosis also occurs but no anti-DNA antibodies are detected. Additionally, injection of nucleosomes into normal mice does not generate the production of anti-DNA autoantibodies or nephritis (80).

## Nucleosome-specific autoantibodies

The central role of nucleosomes for the induction of the autoimmune response in SLE is underlined by the formation of nucleosome-specific antibodies, which react with nucleosomes but not with its components DNA and histones (47). In several lupus prone mouse strains, the previous studies have revealed that antinucleosome antibodies occur early in life, before the emergence of anti-dsDNA and antihistone antibodies (8, 14). This early antinucleosome reactivity was shown, in inhibition and adsorption assays, to be due to antibodies that recognize the native nucleosome particle but not its individual components (DNA and histones). These nucleosome-specific antibodies persist later in the course of disease when anti-dsDNA and antihistone antibodies develop (8, 14). This antinucleosome reactivity was first demonstrated for monoclonal antibodies (mAbs) generated from lupus mice (11), and was also found in a high proportion (> 80%) in the sera of lupus mice and patients, before development of anti-dsDNA and antihistone antibodies (9). The majority of murine nucleosome-specific antibodies belong to the IgG2a and IgG2b subclasses, consistent with a T cell-dependent autoantigen-driven response.

Following structural studies with a growing number of monoclonal anti-dsDNA antibodies have progressed, it has emerged that anti-dsDNA antibodies have

all characteristics of those produced in an antigen-stimulated, secondary immune response (7). They are predominantly of IgG isotype, are highly oligoclonal, and have numerous somatic mutations in their VH regions, features typical of an antigen-driven response (81-83). Monoclonal antihistone autoantibodies have the same properties (84), as do nucleosome-specific antibodies isolated from a single, young, lupus MRL+/+ mouse (a mouse that had serum antinucleosome but no anti-dsDNA nor antihistone antibody titers) at the very onset of the autoimmune response have characteristics of multiclonality and recognize a wide variety of nucleosome epitopes (85). So, these different nucleosome-specific antibody populations could reflect different stages of the autoimmune response. Of many nucleosome-reactive B cell clones recruited at the onset of the autoimmune response in young, predisposed mice (85), only a few may be selected and clonally expanded as the mice age and the disease progresses (86-89).

A footprint of the autoantigen recognized by these antibodies is found in the complementarity determining regions both on the VL and VH parts of molecules. They contain both anionic residues (as in antihistone antibodies) as well as cationic amino-acid residues (as in anti-DNA antibodies), which mediate the binding to the cationic histone or anionic DNA part of the nucleosome, respectively (11, 90). Monoclonal and polyclonal nucleosome-specific antibodies are mainly directed against epitopes within the (H2A-H2B) DNA complex, and to a lesser extent also within the (H3-H4) DNA complex. Some nucleosome-specific antibodies, however, show a higher reactivity to intact nucleosomes than to subnuclear structures, indicating the presence of yet unidentified epitopes (35).

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## Role of nucleosomes in the pathogenesis of SLE

Glomerulonephritis, which ultimately develops in 40-60% of systemic lupus erythematosus patients, is a severe manifestation of the disease with a major impact on morbidity and mortality. Although there is great variability in the histological

findings in kidney biopsies of patients with lupus nephritis, a consistent finding is the presence of all classes of immunoglobulins and early complement factors in glomerular deposits (48). Classically, not nucleosomes but DNA has been thought to play a role in development of tissue lesions in lupus through the formation and subsequent deposition of DNA/anti-DNA immune complex (91). Nevertheless, formal proof for this hypothesis is rather lacking. It has been very difficult to detect circulating free DNA or DNA complexed to anti-DNA antibodies (92-94). Other observation showed that DNA in the circulation of SLE patients was present in the form of (oligo) nucleosomes (1). Following these findings it is likely that DNA is not present in naked form in the circulation but complexed in (oligo) nucleosomes. Additionally, it is difficult to imagine how the anionic charged DNA can have affinity for anionic charged glomerular basement membrane (GBM) (49).

The first notion, that nucleosomes are not only important for the induction of the autoimmune response, but also play a key role in the development of tissue lesions, in particular lupus nephritis, came from experiments with cross reactive anti-DNA antibodies. This observation revealed that anti-DNA antibodies could cross react with an intrinsic component of GBM, namely heparan sulfate (HS), they found that this cross reactivity was not exerted by the antibody itself, but was mediated by nucleosomal material complexed to the antibody (95). HS is responsible for the negative charge of the GBM, and thus for the charge-dependent permeability of the GBM (96). Neutralization of this HS associated charge, or antibody binding to HS leads to albuminuria (97, 98).

The binding of antinuclear antibodies to HS was not due to cross-reactivity, as thought initially, but was mediated by nucleosomes (95). If monoclonal anti-dsDNA antibodies, which reacted in ELISA with HS, were treated with DNAse and were subsequently purified under high salt conditions on a protein-A column, all HS reactivity was lost. Addition of the protein A column effluent restored the binding to HS. Subsequent analysis revealed that histone/DNA complexes (i.e. nucleosomes) were responsible for the binding to HS. Also *in vivo*, in renal perfusion studies in the rat, nucleosomes could mediate the binding to the GBM, while non-complexed anti-nucleosome and anti-dsDNA antibodies did not bind (99).

This nucleosome mediated binding occurred via binding of the cationic N terminal tails of the core histones to the strong anionic charges of HS. Because the epitopes of antihistone antibodies are mainly localized on the N terminal regions, their binding masks the positive charges on these histone tails, thereby preventing binding to anionic HS. These positive charges on the N termini of histones can not be neutralized by the binding of anti-dsDNA or anti nucleosome antibodies. In fact, their binding to the nucleosome has an opposite effect, since they neutralize in part the anionic charges of dsDNA, which makes the complex even more nephritogenic. Using polyclonal and monoclonal antibodies to the various components of the nucleosomes as probes, nucleosomes were indeed identified in human lupus nephritis, predominantly in the diffuse proliferative form (WHO class IV) (100). With elution studies of isolated glomeruli from MRL/*lpr* mice antinucleosome antibodies were identified (101).

A time study revealed that these antinucleosome antibodies were deposited first, with subsequent deposition of anti-dsDNA antibodies (100). This sequence suggests that after lodging of the nucleosome in the GBM, it acts as a planted antigen for subsequent binding of anti-dsDNA. In concordance with the above-mentioned studies that nucleosome/anti-histone complexes are less nephritogenic, the amount of anti-histone antibodies was low and did not increase when the severity of the glomular lesion progressed. An analysis of various glomerular diseases with monoclonal antibodies against HS and the HSPG core protein revealed an almost complete absence of HS staining in the GBM in 90% of the biopsies from patients with proliferative lupus nephritis (102).

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Taken together, these results document the presence of nucleosomes, antinucleosome antibodies, and nucleosome/autoantibody complexes in glomerular deposits in lupus nephritis. They underline the relevance of nucleosome mediated targeting of autoantibodies to the GBM, as identified in experimental animal studies. The summary of the mechanisms leading to autoimmunity to nucleosomes in SLE and the development of lupus nephritis is graphically depicted in Figure 5 (67). Because of their nephritogenic potential, it could be helpful to identify nucleosome/ autoantibody complexes in the circulation of SLE patients. Using an ELISA, plasma samples of SLE patients were screened for anti-HS reactivity. Onset (103) or

exacerbation (104) of lupus nephritis was indeed associated with higher anti-HS reactivity. Using a GBM based ELISA similar results were found (105).

Nonetheless, other studies with MRL/*lpr* and human SLE sera have identified type IV collagen, another major component of the GBM, as a candidate ligand for nucleosome/antinuclear immune complexes (106, 107). Collagenase treatment of the GBM prevented the binding of nucleosome/IgG complexes, suggesting that IV collagen was important as a ligand. Taken together, these findings have identified a similar binding mechanism of nucleosome/antinuclear complexes to GBM via either HS or IV collagen. This can explain why, in the renal perfusion system, the removal of HS by heparinase reduced but not completely abrogated binding (99).

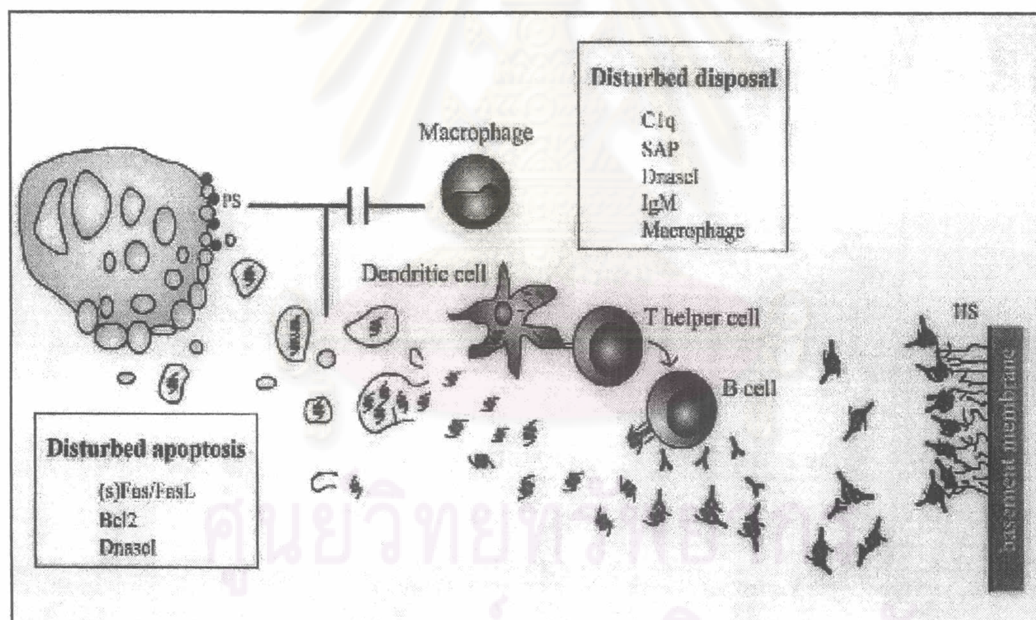


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**Figure 5:** The summary of the mechanisms leading to autoimmunity to nucleosomes in SLE and the development of lupus nephritis.

**Clinical relevance of antinucleosome antibodies**

Although the nucleosome has been identified as a major immunogen in SLE, antinucleosome-specific antibodies are not tested routinely in lupus patients. There are now indications that this broad antibody population could be clinically relevant. First, the nucleosome (or chromatin) is responsible for the most reactive substrate among the nuclear antigens, 55-80% of SLE patients being positive (15, 18, 21, 22, 107, 108). Second, independent studies have demonstrated that the contribution of anti-dsDNA and antihistone antibodies to serum reactivity against nucleosomes in SLE patients is only 25-30% at most (18, 21). Third, approximately one-third of SLE sera studies have high antichromatin/nucleosome activity and little if any anti-dsDNA or antihistone reactivity (16, 18, 21).

In fact this antinucleosome antibodies is potentially nephritogenic, its titer correlates with the Systemic Lupus Erythematosus Activity Index score, a validated index of SLE activity, and these antibodies were found to be more highly associated with nephritis than anti-dsDNA antibodies (15, 17). In a cross-sectional study of 120 SLE patients with active and inactive disease (22), Amoura et al showed that 65% of the anti-dsDNA-negative sera had antinucleosome activity and a similar results was demonstrated in recent study by Min et al (17). Thus these data suppose the view that antinucleosome antibodies might be a useful marker of anti-dsDNA negative SLE. Furthermore Min et al also showed that of the patients with anti-dsDNA negative SLE, renal disorders were found in 32% and all sera had antinucleosome antibodies (17). In the same study they demonstrated the levels of antinucleosome antibody strongly correlated with the disease activity (SLEDAI) index, but conversely correlated with complement levels in anti-dsDNA negative SLE patients.

The prevalence of antinucleosome antibodies in lupus mice and patients are summarized in Table 5. The studies revealed in table 5 shown a strong correlation between antinucleosome reactivity and disease activity and lupus nephritis. In one study they examined the prevalence of antinucleosome IgG in 13 connective tissue diseases and found that its presence was limited to SLE, scleroderma, and mixed

connective tissue disease (22). This suggests that antinucleosome antibodies could be most helpful in the differential diagnosis of connective tissue diseases. Moreover, in the same study, antinucleosome antibodies of the IgG3 subclass were exclusively detected in SLE and specifically related to renal flares. A similar correlation was not found for IgG3 anti-dsDNA (22). According to these data, it is clear that measurement of antinucleosome reactivity is preferable and more specific than anti-dsDNA.

**Table 5:** Prevalence of antinucleosome reactivity in SLE

| Reference | Analysed population | Prevalence (%) | Characteristics |
|---|---|---|---|
| Fisher et al (1988) (109) | MRL/lpr mice (n>500) | 100 | Increasing titer with age<br>50% IgG2a, 30% IgG2b, 10% IgG1, 10% IgG3 |
| | MRL/+ mice<br>(NZBxNZW)F1<br>BxSB<br>control strains | 72<br>61<br>69<br>0 | Subclass distribution similar and titers lower than in MRL/lpr mice |
| Burlingame et al (1994)(21) | SLE patients (n=40) | 88 | Anti-chromatin and anti-H2A-H2B/DNA correlated with lupus nephritis |
| Suenaga et al (1996)(110) | SLE patients with nephritis (n=6) | 60 | |
| Amoura et al (2000)(22) | systemic autoimmune disease (n=496)<br>chronic hepatitis C infection (n=100)<br>healthy controls (n=406)<br>Controls: 0 | SLE: 72<br>SSC: 46<br>MCTD: 45 | IgG3 antinucleosome antibodies exclusively present in SLE and related to lupus nephritis and disease activity (SLEDAI) |
| Bruns et al (2000)(15) | SLE patients (n=106)<br>other systemic autoimmune diseases (n=26)<br>healthy controls (n=105) | SLE: 56<br>All others: 3 | - Better sensitivity and specificity than measurement of anti-dsDNA (diagnostic confidence antinucleosome: 90%. anti-dsDNA: 69%)<br>- antinucleosome correlated significantly with disease activity (ECLAM), lupus nephritis and psychosis |
| Min et al (2002)(17) | SLE patients (n=129)<br>healthy controls (n=50) | SLE : 76 | - antinucleosome were present in 60% of the anti-dsDNA negative SLE, renal disorders were present in 32% in this group and all of whom had antinucleosome antibodies<br>- levels of antinucleosome strongly correlated the SLEDAI scores |

Abbreviations: SSC=systemic sclerosis; MCTD=mixed connective tissue disease; SLEDAI=SLE disease activity index; ECLAM=European Consensus Lupus Activity Measurement Index.

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