M.Sci. Thesis in Molecular Immunology

Analysis of glomerular target structures for nephritic anti-dsDNA antibodies playing central roles in lupus nephritis

By

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2008

ACKNOWLEDGEMENTS

The present work was performed at the Department of Biochemistry, Institute of Medical Biology at the University of Tromsø, in the period of August 2006 to May 2008.

First of all I would like to thank my main supervisor Kristin Andreassen Fenton for positive attitude, enthusiasm and a lot of patience when I lost faith. Her belief in the project and open minded approach to scientific problems was a great motivation factor. I would like to thank my second supervisor Ole Petter Rekvig as well, for standing behind it all with great knowledge and for suggestions and critical comments to my manuscript.

Thanks to Dr. Tony Marion, Memphis, Tennessee for kindly providing the hybridoma cell lines.

I also wish to thank Randi Olsen, Jørgen Benjaminsen and Helga Marie Bye at the Electron Microscope Department for technical contribution, and support when running the microscope, and the staff at the Department of Animal Research for regularly taking blood samples of the mice.

I am also very grateful to Trine Tessem and Kari Haugli at the office for support, encouragement and inspiration, and Ellen Valberg for fun and friendship and help when the computer was on its way out the window. You have been irreplaceable!

Thanks to all in the group for a friendly and including atmosphere at the laboratory and also former colleges at the institute, and Manar Kalaaji for believing in me.

Last but not least, I will especially thank dear friends and my mother for always being there.

Tromsø, May 2008

Berit Tømmerås

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ABSTRACT

Systemic lupus erythematosus is an autoimmune disease characterized by the presence of autoantibodies towards nuclear antigens. Sub-populations of anti-dsDNA antibodies have the potential to bind in the kidney and thereby induce nephritis, but there is no definite distinction that separates non-pathogenic from pathogenic antibodies. There must be parameters that determine anti-dsDNA antibody binding, and properties as high affinity and specificity for dsDNA, cross-reactivity with inherent renal molecules, and availability for target antigens have been suggested as possible candidates.

In this study pre-characterized sub-populations of purified monoclonal anti-dsDNA antibodies (mAbs) were injected into BALB/c mice. To trace the glomerular targets for the antibodies, immunological microscopy at an ultrastructural level were performed, and proteinuria and serum levels of mAbs were monitored.

The attempt to reveal structural differences of mAbs that correlate with differences in antigen binding failed. Serial injections of all mAbs showed binding to matrix components and mesangial sites of glomerular basement membrane (GBM). There were no properties that stand out as responsible for this; all mAb had different molecular features and combinations of these. Second, all mAbs showed high affinity for dsDNA *in vitro* and all was binding *in vivo.* These facts points at affinity as a poor marker for pathogenicity. It seems like anti-dsDNA antibodies in general, and not specific sub-populations, are involved in development of glomerulonephritis.

The availability of antigens might be the important parameter. All mAb showed cross-reactivity toward inherent antigens *in vitro*, but this does not by itself indicate pathogenesity as no binding to these inherent antigens was demonstrated *in vivo.* Stronger evidence points at chromatin as the main antibody-binding target, considered it is available. All tested antibodies showed specificity for nucleosomes, which is the natural preferred target, and EDS in glomeruli showed strictly co-localization of anti-dsDNA antibodies and the presence of DNA.

ABBREVIATIONS

Ab	antibody
β2GPI	β2 glycoprotein I
BSA	bovine serum albumin
CDR	complementary-determining region
CIEM	co-localization IEM
Dialysate	dialysed mAb preparations
DIF	direct immunofluorescence microscopy
DNA	deoxyribonucleic acid
dsDNA	double stranded DNA
EDS	electron dense structures
ELISA	enzyme-linked immunosorbent assay
EM	electron microscopy
FBS	foetal bovine serum
GBM	glomerular basal membrane
HS	heparan sulphate
HSPG	heparin sulphate proteoglycans
IEM	immune electron microscopy
Ig	immunoglobulin
kb	kilo base
kDa	kilo Dalton
LN	lupus nephritis
mAb	monoclonal antibody
NZB/W	(NZB x NZW)F1 mice
OD	optical density
PAG	protein A gold
PBS	phosphate buffered saline
PC	phosphatidylcholine
PS	phosphatidylserin
SDS-PAGE	sodium dodecyl sulphate – polyacrylamide gel electrophoreses
SLE	systemic lupus erythematosus
SPR	surface plasmon resonance
ssDNA	single stranded DNA
TEM	transmission EM

1 Introduction

1.1 The immune system

A normal immune response is directed against foreign antigenic substances as microorganisms, and the normal consequences are clearance of the antigen from the body. Encountered with the antigen, the immune system initially responds with the production of antibody molecules specific for the immunogen and a state of lymphocyte memory develops. That means that a second intruding of the same antigen will trigger the immune system to a more rapid and more effective response to clear the antigen from the body.

The immune system is highly specific, and has a unique ability to discriminate between antigenic epitopes expressed on foreign substances and antigenic epitopes expresses by the host. Elimination or inactivation of lymphocytes that recognizes self-antigens ensures non-responsiveness to self- antigens, and is called immunologic tolerance. One basic factor for inducing an immune response is the mutual costimulating of lymphocytes. For B lymphocytes (B cells) to develop into antibody producing plasma cells, the first stimulus come from the antigen itself, and the second stimulus are provided by activated T lymphocytes (T cells). These also acquire a first signal from antigen through the receptor complex, and are further stimulated by antigen presenting cells (APC) which can be B cells among others (Janeway et al., 2005).

1.2 Structure of IgG antibodies

The IgG molecule consists of two classes of polypeptide chains, light chains and heavy chains, and has two identical copies of each. This makes the IgG antibody molecule a four chain structure comprised of a variable (V) and a constant (C) region. The V- region of one heavy and one light chain together make an antigen binding domain, and are composed of an infinite variety of different amino acid sequences, forming different three-dimensional structures allowing the molecule to bind specifically to homologous antigens. The IgG antibody is bivalent; it has two of these binding sites for antigen (Figure 1.1).

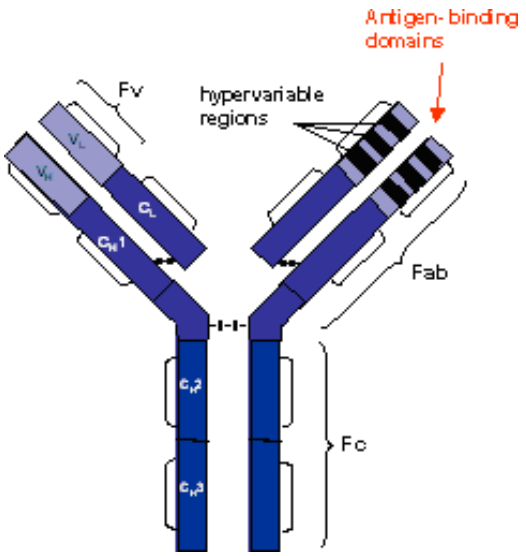

Figure 1.1. Schematic diagram of an immunoglobulin molecule. The immunoglobulin molecule comprises two of each type of light (L) and heavy (H) chain, which are covalently linked by disulphide bonds. The antigen binding domains are formed by hypervariable regions of the heavy and light chains, and comprise four framework regions and three complementarily-determining regions (CDR1, CDR2 and CDR3). The heavy-chain constant region is made up of three constant domains (C_H1, C_H2 and C_H3) and the flexible hinge region. The light chain also has a constant domain (C_L). (www.affimed.com/images/diag1.gif)

The great diversity is generated by random recombination of separate gene segments (V, J, D for heavy chain and V, J for light chain) which is presented at multiple versions. Imprecise joining of the segments, random pairing of heavy and light chains and somatic mutations also contributes to diversity basically outlined in Figure 1.2

The antigen contact domains at the V-region are highly polymorphic and are known as complementary-determining regions (CDR). Three CDRs are separated by four framework regions at each chain, and CDR3 at the D-segment of heavy chain are the most versatile segment. These hyper variable segments are arranged in flexible loops at the tip of the V-regions that together form a surface that is complementary to the three-dimensional structure of the bound antigen.

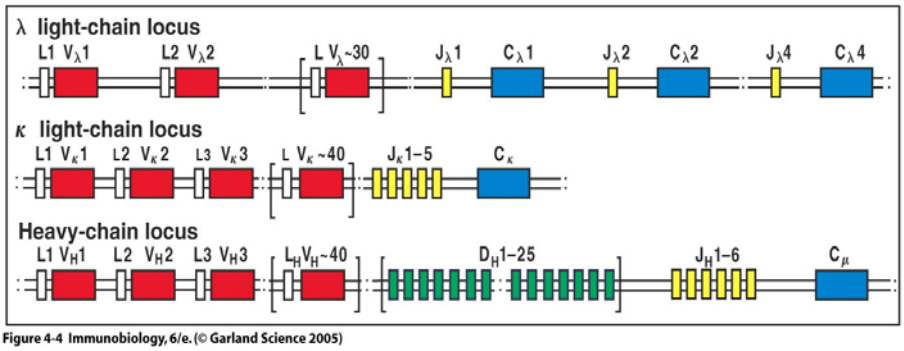

Figure 1.2. The genomic organization of the immunoglobulin heavy and light chain gene segments in human genome. The genetic locus for the λ light chain has about 30 functional V_λ gene segments and four pairs of J_λ gene segments and C_λ genes. The alternative κ light chain locus is organized the same way, with 40 Vκ gene segments and a cluster of five Jκ gene segments, but a single Cκ gene. The heavy chain locus has about 40 functional V_H gene segments, 25 D_H segments, six J_H genes and a large cluster of C_H genes (Janeway et al., 2005).

Immunoglobulins are made in several distinct isotypes or classes, as immunoglobulin (Ig) G (IgG), IgM, IgE, IgD and IgA. Each is determined by a distinct heavy chain C-region, and performs different set of effector functions. The C-region of IgG antibodies mediates effector functions such as binding to Fc-receptors on phagocytic cells and activation of the complement system. Additional structural variations characterize four distinct subtypes of IgG, IgG 1-4 (Janeway et al., 2005)

1.3 Tolerance

The immune system is constantly exposed to self-antigens without stimulating lymphocytes. Mature lymphocytes recognize and respond to foreign antigens, but ignore molecules from the body itself. This phenomenon is called self-tolerance. This is maintained by mechanisms at different levels that prevent the maturation and activation of potentially harmful self-reactive lymphocytes.

Thymus and bone marrow are primary lymphoid organs where precursor cells differentiate into respectively mature T and B cells. These are also the sites where self antigens are present at high concentrations. When immature T and B lymphocytes encounter self antigens with high affinity, they are trigged to undergo apoptosis; they are deleted, and this is called central tolerance. However, mature self reactive T and B lymphocytes are present in the circulation of healthy individuals (Avrameas, 1991), indicating that clonal elimination is incomplete. To avoid further expansion of these potentially autoimmune B and T cells, there are other regulatory mechanisms.

For a normal immune response to occur, the B and T cells need two distinct sets of extracellular signals to be activated. For both types of lymphocytes, signal 1 is provided by antigen recognition and is insuring that the immune response is specific. Signal 2, is represented by the co-stimulatory signal, and are delivered by antigen presenting cells (APC), including B cells. If mature lymphocytes recognize self-antigens in peripheral tissue, in absence of adequate co-stimulation, the lymphocytes enter a state of unresponsiveness called anergy; peripheral tolerance is induced (Reeves et al., 2004; Amital and Shoenfeld, 2004).

1.4 Autoimmunity

Autoimmunity is defined as adaptive immune responses to self antigens (autoantigens) because of loss of self-tolerance. This means that the immune system turns against self antigens and attacks the body's own tissue.

Every individual have the potential for autoimmunity. B and T lymphocytes with an infinity amount of different specificities including self-reactive, are constantly made as part of the pool of diversity. When the selection process that silences the self-reactive lymphocytes fail, surviving self-reactive lymphocytes is stimulated by the homologous autoantigen and autoantibodies are produced. For an autoimmune disease to be clinical apparent, additional parameters are important including genetic, hormonal and environmental conditions (Amital and Shoenfeld, 2004).

Autoimmune diseases can broadly be divided into organ-specific and systemic autoimmunity. In the former, the immune response is directed against a particular organ and the damage is limited to that given organ. The latter case is associated with the appearance of antibodies against ubiquitous antigens such as chromatin and other molecules present in all nucleated cells. Lack of specificity for any given organ may lead to multiple organ affection, and the disease often becomes chronic. Because the adaptive immune response is incapable of removing the autoantigen from the body there is a constant supply of autoantigens, and the immune response will persist (Amital and Shoenfeld, 2004).

1.5 Systemic lupus erythematosus

Systemic lupus erythematosus (SLE) is a chronic, systemic, autoimmune disorder. The prevalence is 1:2000 to 1:10.000 in the general population. Individuals from all ages, countries and both sexes are affected, and the highest incidence is among females of child bearing age and among afro-American minorities (Lahita, 2004).

This systemic disease may affect a wide array of organs, from skin and mucous membranes to central nervous system, joints, kidneys and other. The clinical manifestations are highly diverse and variable, and result from interplay between multiple factors that vary in participation among patients. Different diagnostic criteria have been summarized by the American College of Rheumatology (ACR) for classification (Table 1.1) (Tan et al., 1982). Four or more of these criteria must be fulfilled, serially or simultaneously, to set the diagnosis

of SLE. However, it is at present ongoing discussions whether SLE with different clinical manifestations constitutes distinct entities or variant expressions of the same disorder (Rekvig and Nossent, 2003).

Typical for the disease is inflammation of different organs. This is partly mediated by autoantibody deposits. Production of antibodies against a wide variety of self-antigens is characteristic for SLE, primarily against nuclear antigens. Although a cell nucleus contains a large number of different molecules, few are autoantigens in systemic autoimmune diseases. Production of antinuclear antibodies (ANA) is a main feature of SLE, and is included in the ACR classification as criterion 11. Some of the ANAs can have pathogenic potential. For instance, antibodies to dsDNA (ACR classification criterion 10) are often associated with the renal disorder glomerulonephritis (ACR classification criterion 7). See Table 1.1 (Tan et al., 1982).

1.6 Origin of anti-dsDNA antibodies

Anti-dsDNA antibodies were first identified in 1957 (Robbins et al., 1957; Ceppellini et al., 1957). Despite intense studies the last fifty years, the origin of anti-DNA antibodies is still not fully understood. Factors leading to their production include break of B-and T cell tolerance, with the consequence of autoimmunity to nucleosomes and particularly to the individual components as native (ds) DNA and histones (Reeves et al., 2004; Rekvig and Nossent, 2003)

Naked DNA is a poor immunogen, and its immunogenicity is believed to depend on complex formation with immunogenic peptides. Different studies have demonstrated that antibody to dsDNA can be induced by experimental immunization with dsDNA, provided it is in complex with an immunogenic carrier protein (Rekvig and Nossent, 2003; Desai et al., 1993; Pisetsky and Drayton, 1997) or by truly autoimmune nucleosome specific T cells (Mohan et al., 1993; Andreassen et al., 2002) The multivalent nature of these linked sets are able to stimulate dsDNA-specific B cells and peptide-specific T cells in analogy to the classical hapten-carrier model for induction of anti-hapten antibodies (Figure 1.3) (Desai et al., 1993; Rekvig et al., 1997a; Mohan et al., 1993; Datta et al., 2005; Andreassen et al., 1999b; Bendiksen et al., 2004)

Table 1.1. The criteria of the American College of Rheumatology (ACR) for the diagnosis of systemic lupus erythematosus*

Criterion	Definition
1. Malar rash	Fixed erythema, flat or raised, over the eminence, tending to spare the nasolabial folds
2. Discoid rash	Erythematosus raised patches with adherent keratotic scaling and follicular plugging; atrophic scarring may occur in older lesions
3. Photosensitivity	Skin rash as a result of unusual reaction to sunlight, by patient history or physician observation
4. Oral ulcers	Oral or nasopharyngeal ulceration, usually painless, observed by a physician
5. Arthritis	Nonerosive arthritis involving two or more peripheral joints, characterized by tenderness, swelling or effusion
6. Serositis	a) Pleuritis: convincing history of pleuritic pain or rub heard by physician or evidence of pleural effusion OR b) Pericarditis: documented by ECG or rub or evidence of pericardial effusion
7. Renal disorder	a) Persistent proteinuria greater than 0.5 g per day or greater than 3+ if quantitation not performed OR b) Cellular casts: may be red cell, hemoglobin, granular tubular, or mixed
8. Neurologic disorder	a) Seizures: in the absence of offending drugs or known metabolic derangement; e.g., uremia, ketoacidosis, or electrolyte imbalance OR b) Psychosis: in the absence of offending drugs or known metabolic derangement, e.g., uremia, ketoacidosis, or electrolyte imbalance
9. Hematologic disorder	a) Hemolytic anemia: with reticulocytosis OR b) Leukopenia: less than $4000/mm^3$ total on two or more occasions OR c) Lymphopenia: less than $1500/mm^3$ on two or more occasions OR d) Thrombocytopenia: less than $100,000/mm^3$ in the absence of offending drugs
10. Immunologic disorder	a) Anti-DNA: antibody to native DNA in abnormal titer OR b) Anti-Sm: presence of antibody to Sm nuclear antigen OR c) Positive finding of anti-phospholipid antibodies based on (1) an abnormal serum level of IgG or IgM anti-cardiolipin antibodies, (2) a positive test result for lupus anticoagulant using a standard method, or (3) a false positive serologic test for syphilis known to be positive for at least six months and confirmed by *Treponema pallidum* immobilization or fluorescent treponemal antibody absorption test.
11. ANA	An abnormal titer of ANA by immunofluorescence or an equivalent assay at any point in time and in the absence of drugs known to be associated with "drug-induced" lupus syndrome

* The classification is based on 11 criteria. For the purpose of identifying patients in clinical studies, a person must have systemic lupus erythematosus if any 4 or more of the 11 criteria are present, serially or simultaneously, during any interval of observation.

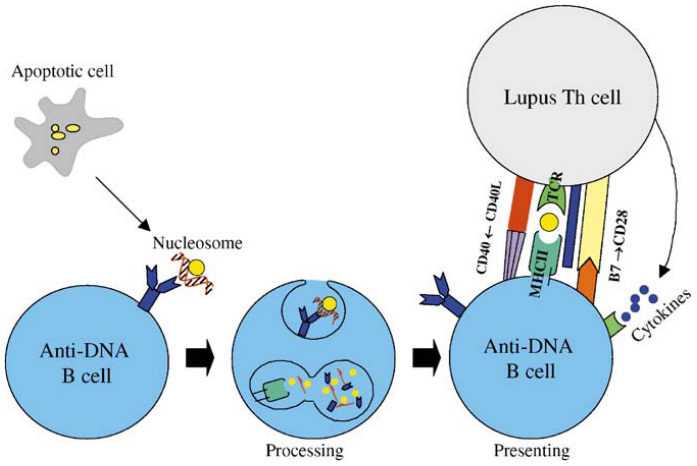

Figure 1.3. Processing and presentation of nucleosomes for autoimmune T cells.
DNA-specific B cells recognize and internalize apoptotic nucleosomes, and process and present it to nucleosome-specific T cells. For both types of lymphocytes, signal 1 is provided by antigen recognition and is insuring that the immunresponse is specific. Upon interaction with antigen, antigen presenting cells (APCs), such as B cells and macrophages are stimulated to express co-stimulatory surface molecules not present on resting APCs. Among these, the B7 molecules play an important role in the generation of T cell activation. The interaction is bidirectional; the co-stimulatory molecule CD40L is expressed on activated T cells, and is together with cytokines, responsible for B cells differentiating into antibody (including autoantibody) secreting plasma cells (Datta et al., 2005)

An alternative model for inducing autoimmunity is Matzinger's danger model. It suggests that the autoimmune response is trigged by endogenous alarm signals (as nucleosomes) from damaged tissue and by uncleared apoptotic cells in the individual. These signals activate antigen presenting cells, necessary for presenting co-stimulatory signals to T cells. These self molecules are normally hidden from the immune system, but when exposed because of disintegrated membranes, they may reveal structures that trigger immune response. The superior message in this model is the important discrimination between potential danger to the individual or not. If this danger is from self or non-self are of minor importance (Matzinger, 2007; Matzinger, 2002)

From analysis of the V-region structure of monoclonal anti-dsDNA antibodies, it is suggested that as the autoimmune response progress, the antibodies maturate towards the driving antigen with increased affinity, a process called affinity maturation (Marion et al., 2003; Krishnan and Marion, 1998; Radic et al., 1993) Anti-ssDNA is naturally found in the body, and is present at low titers and is of IgM type. Studies have shown that as the autoimmune response progresses, the antibody isotype switches to IgG, and the antibody acquires affinity for dsDNA (Marion et al., 1992; Tillman et al., 1992; Rekvig and Nossent,

2003). This is a natural ongoing process based on multiple somatic mutations of immunoglobulin V-region genes and is followed by positive selection of the B cells producing antibodies with the highest affinities As the somatic mutations accumulate, change in specificity may occur. Depending on the property of the new amino acids introduced in the variable CDR regions of the B cell receptor, the antibody may switch the specificity from ssDNA towards dsDNA (Tillman et al., 1992; Marion et al., 1989). Especially introduction of arginine in the CDR3 region of heavy chain is demonstrated to gain affinity for dsDNA (Krishnan and Marion, 1993).This progressive development from lower affinity IgM anti-ssDNA antibodies towards higher affinity IgG anti-dsDNA antibodies is referred to as affinity maturation. (Figure1.4)

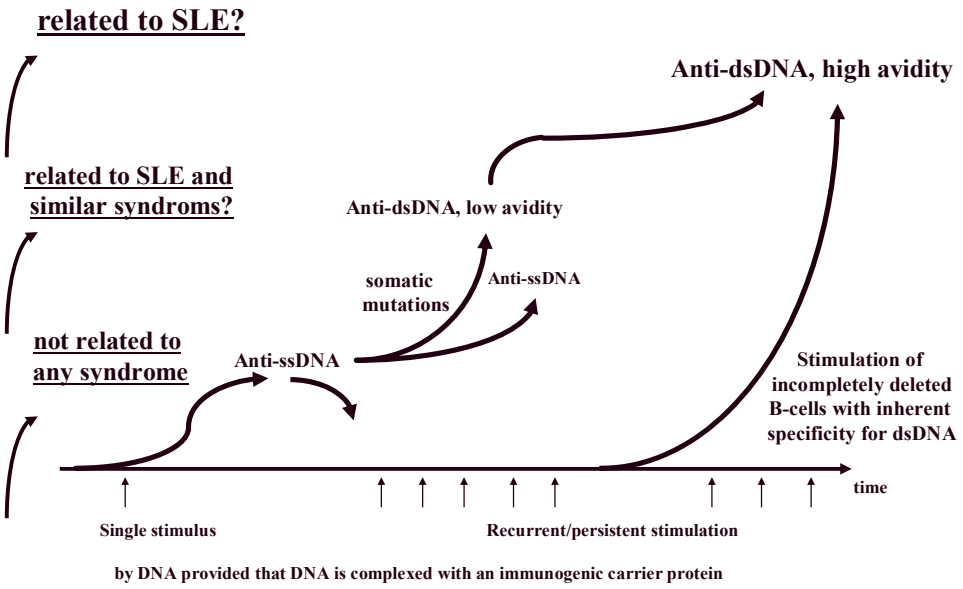

Figure 1.4. Two pathways for generation and selection of anti-dsDNA antibodies. Two models are suggested for activation of DNA-specific B cells resulting in production of high affinity anti-dsDNA antibodies: Stimulation of incompletely deleted B cells with inherent specificity for dsDNA eventually followed by affinity maturation, or stimulation of B cells with specificity for ssDNA followed by somatic mutation as the immune response progress (Rekvig and Nossent, 2003).

1.7 Disturbed apoptosis and nucleosomes as autoantigen

As described above, nucleosomes rather than dsDNA might be the driving autoantigen in SLE, and dying cells are the only source for nucleosomes. Apoptosis is a tightly regulated process of programmed cell death and is characterized by enzymatic fragmentation of cell contents, including chromatin (Savill, 2000; Casciola-Rosen et al., 1994). The nucleosome is the basic unit of chromatin, and is composed of DNA, histones and other proteins. Through the combination of cationic and anionic structures, the overall charge is neutral. The core is an octamer containing two copies each of histones, H2A, H2B, H3 and H4, and about 146 base pairs of dsDNA wrapped about 1 $^{3}/_{4}$ turns around. Arginine side chains of the histone proteins are responsible for anchoring the double helix to the core, and the structure is further stabilized by histone H1 at the linker DNA region outside the nucleosome (Figure 1.5) Linker DNA, which is exposed, is degraded by nuclear endonucleases in order to produce oligo-nucleosomes and mono-nucleosomes (Decker, 2006).

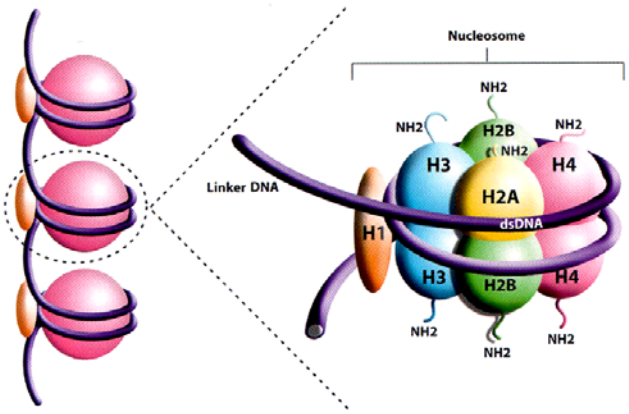

Figure 1.5. Schematic representation of a nucleosome. The nucleosome consists of a core particle composed of an octamer of histones and a 146 base pair stretch of DNA coiled around the complex. (D.Villalta and R. Tozzoli, Autoantibodies 2nd edition)

After initiated cleavage of chromatin, there are a number of surface changes in apoptotic cells. In contrast to phosphatidylcholine in the outer membrane leaflet of healthy cells, the intracellular phosphatidylserine are turned inside out and are expressed on the surface of the apoptotic cell and apoptotic blebs. This acts as an 'eat me' signal and serves as recognition molecules for scavenger cells (Herrmann et al., 1998). If clearance of apoptotic cells are interrupted, the dying cell may go through secondary necrosis; get further

enzymatically destroyed and release high amount of modified nuclear and cytoplasmic material (Gaipl et al., 2005), including their chromatin (Herrmann et al., 1998; Savill et al., 2002).

Modification of autoantigen during abnormal apoptosis can make them more immunogenic (Rosen and Casciola-Rosen, 1999). Cryptic autologous epitopes may be exposed to the immune system as foreign epitopes (Utz and Anderson, 1998; Koutouzov et al., 2004; Hall et al., 2004) and cross-reactive epitopes may rise from the conformal position in the tertial structure of the target protein within nucleosomes (van Bavel et al., 2007).

Dysregulation of apoptosis together with defects in clearance of dying cells have been described in both murine and human SLE (Berden, 2003; Dieker et al., 2004; Makino et al., 2003; Perniok et al., 1998) and an increased number of apoptotic cells in glomerulus of NZB/W mice (Kalaaji et al., 2006a) and human SLE patients (Kalaaji et al., 2007) have been reported. This leads to an increased release of autoantigens, and have been implicated in the pathogenesis of lupus nephritis (Berden et al., 2002; Furnrohr et al., 2007; Gaipl et al., 2005; Herrmann et al., 2000). Approximately, in 40% of patients with SLE, there is an impaired clearance of apoptotic cells (Herrmann et al., 2000).

Immune complexes are formed by antibodies and their corresponding antigens. Several studies have demonstrated that nucleosomes may be the in vivo target of anti dsDNA antibodies (Burlingame and Rubin, 1990; Chabre et al., 1995; Kalaaji et al., 2007). Apoptotic nucleosomes may represent both inducer and target structures for nephritogenic autoantibodies in SLE.

1.8 Lupus nephritis

Inflammation affecting the kidneys is one of the most serious manifestations of SLE. Lupus glomerulonephritis, or lupus nephritis, refers to inflammation of the glomeruli of the kidneys.

Glomeruli are structures composed of arterial capillaries that are responsible for filtering the blood for harmful or unnecessary ions and molecules. These vessels are built of a glomerular basement membrane (GBM), with endothelial cells on the blood side and podocyttes (epithelial cells) on the urine side. Through the walls, water and molecules of a certain size can pass, and the unit functions as a biological filter (Figure1.6).

Basement membranes are specialized extracellular matrix found throughout the body, and are especially important in the kidneys, and serve a critical role for filtration function. The

major components of the GBM are laminin, collagen IV, entactin/nidogen and sulphated proteoglycans. The latter consists of protein cores with negatively charged side chains of heparan sulphate (Miner, 1999) and are called heparin sulphate proteoglycans (HSPG). The GBM contains a dominant HSPG called agrin and the mesangial matrix contains a HSPG called perlecan.

Mesangial cells and their surrounding matrix are sited at the interior of the glomerulus (Figure 1.8) and possess a heavy array of microfilaments composed of actin, α-actinin and myosin. The contractile mesangial processes appear to bridge the gap in the glomerular basement membrane encircling the capillary, and also prevent capillary wall distension when elevation of hydraulic pressure as well as participating in control of filtration. This cell also exhibit phagocytic properties and uptake of immune complexes. The mesangial matrix is similar to GBM and contain, additional to HSPG, fibronectin, laminin and collagens (Madsen and Tisher, 2004)

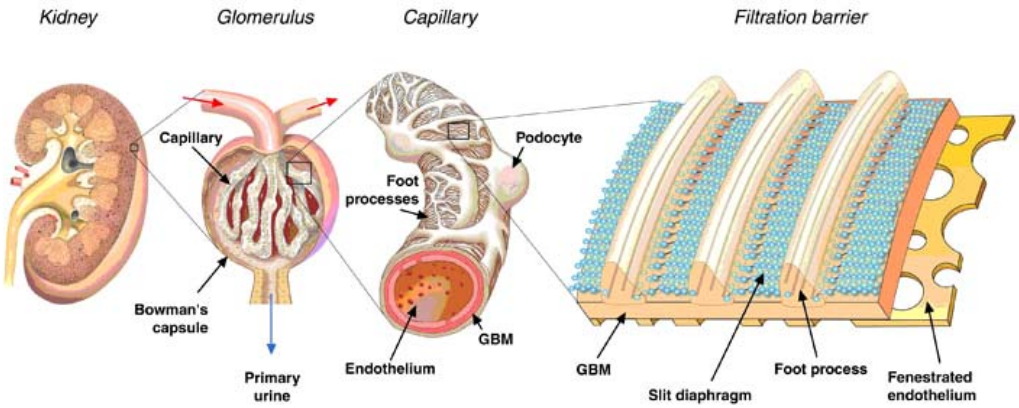

Figure 1.6. Vertical section through a normal kidney and the filtrating unit.
(www.academic.kellogg.cc.mi.us)

Inflammation of glomerulus reduces the ability to clear water and molecules out of the bloodstream, and the damage also allows serum protein as albumin, to leak into the urine space and this is referred to as proteinuria. Lupus glomerulonephritis develop in about 50% of SLE patients (Weening et al., 2004; Balow et al., 2004) and 20% of these individuals get the end-stage renal failure (Nossent et al., 1990)

Lupus nephritis is mediated by immune complex (formed by antibodies and their corresponding antigens) deposits, but this alone is insufficient for the manifestation of the disease. For the pathogenicity, activation of effector mechanisms is required. This is largely influenced by the complement cascade, a system of plasma proteins that interacts with

antigens to mark them for destruction. Complement activation are initiated by binding of C1q, the first protein in the classical complement pathway to the Fc region of the antibody part of the immune complex. The consequence is generation of chemotactic molecules followed by influx of phagocytic cells, release of proteolytic enzymes and destroyed tissue. In addition, stimulation of leucocytes via Fc receptors triggers different responses as degranulation or release of cytokines and proteolytic enzymes and culminating in tissue damage. In contrast, direct phagocytosis of apoptotic cells will have an anti-inflammatory effect (Wener and Mannik, 2004).

In kidney biopsies of SLE patients a great variety of different abnormalities can be present. Based on this, the different stages of morphological degradation are systematically described in the World Health Organization classification of lupus nephritis (Table 1.2) (Balow et al., 2004).

Table 1.2. World Health Organization Classes (WHO) of Lupus Nephritis (Balow et al., 2004)

I.	Normal, or minimal glomerular abnormality, no glomerular abnormalities on light microscopy
II.	Mesangial nephropathy Mesangial expansion, including hypercellularity, increased matrix, and immune complex deposits
III.	Focal proliferative glomerulonephritis Predominantly segmental hypercellularity ± necrosis compromising the circulatory space of capillary loops in ≤50% of glomeruli; mesangial and subendothelial immune complex deposits
IV.	Diffuse proliferative glomerulonephritis, characteristically global but irregular hypercellularity, ± necrosis, ± cellular crescents affecting > 50 % of glomeruli; variable sclerosis, atrophy, fibrosis; mesangial and subendothelial (± subepithelial) immune complex deposits
V.	Membranous nephropathy Generalized thickening of capillary loops; mesangial and subepithelial immune complex deposits
VI.	Sclerosing nephropathy Predominantly hyalinized glomeruli, tubular atrophy, interstitial fibrosis; no (or very rare) immune complex deposits

According Table 1.2, deposits of immune complexes have been identified in all stages of lupus nephritis (except final stage), and may be located to mesangial, subendothelial and subepithelial compartments of the glomeruli (Figure 1.7) (Wener and Mannik, 2004). The anatomic location of the immune deposits may contribute to the development and clinical manifestations of the disease (Waldman and Madaio, 2005; Couser, 1998; Wener and Mannik, 2004). In electron microscopy, these deposits take form as fingerprint-like structures, and are referred to as electron dense structures (EDS) (Hvala et al., 2000).

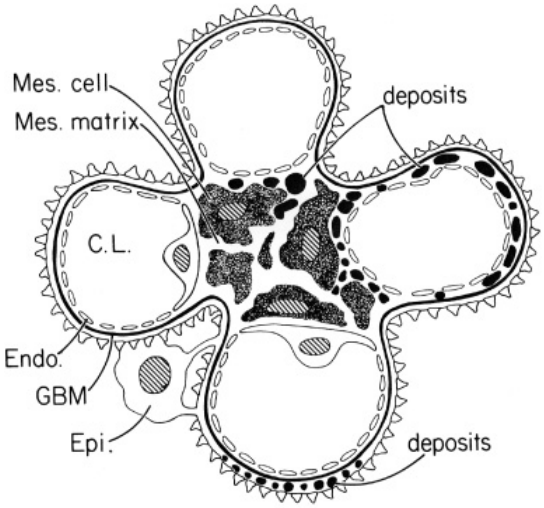

Figure 1.7. Cross section of glomerular capillary lumen (CL) showing location of immune complex deposits. The glomerular basement membrane (GBM) is lined with fenestrated endothelial cells (Endo) on one side and foot processes of epithelial cells (Epi) on the other side. The area between the capillary loops is the mesangium, consisting of mesangial matrix (Mes.matrix) and mesangial cells (Mes.cell). Taken from (Wener and Mannik, 2004).

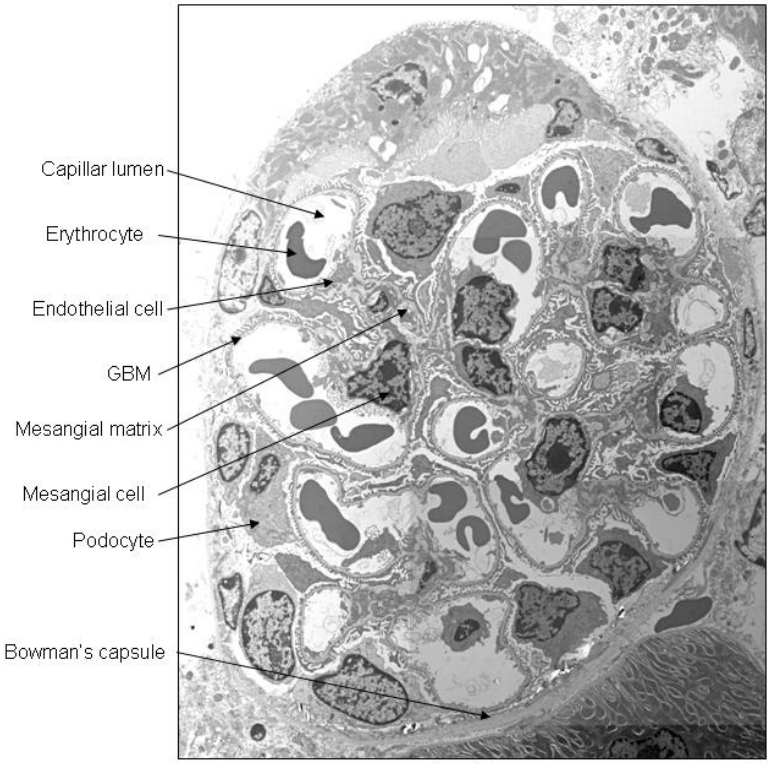

Figure 1.8. Cross section through a glomerulus from BALB/c mouse. Transmission electron microscopy demonstrating major components of a glomerulus from a BALB/c mouse. The diameter of the glomerulus is approximately 50µm. (Micrograph by Fenton, 2007)

1.9 Pathogenesity of anti-dsDNA antibodies

The pathogenic potential of anti-dsDNA antibodies depends on their structural and molecular properties. Nephritic anti-dsDNA antibodies have high affinity towards dsDNA, and are shown to be of IgG isotype that fix complement (Foster et al., 1993; Hahn, 1998). Sub-populations of anti-dsDNA are identified to have defined molecular specificity, and these properties have been thought to determine whether they bind targets in glomerulus (Gilkeson et al., 1995; Vlahakos et al., 1992; Tsao et al., 1992)

Antibody-antigen intermolecular forces are referred to as 'affinity'. Affinity maturation is based on the accumulation of basic amino acids residues in the CDR3 segment of the heavy chain of the V-region, and anti-dsDNA antibodies have increased numbers of such amino acids (Krishnan and Marion, 1993; Rekvig et al., 1995; Tillman et al., 1992). Arginine is a cationic amino acid that has the potential to react with the negatively charged phosphodiester backbone of DNA, or donate up to five H-bonds upon binding to nucleotides (Eilat et al., 1988; Radic and Weigert, 1994; Marion et al., 1992). Mutation experiments have shown that replacement of arginine residues into glycine in the CDR3 abolished the binding activity (Radic et al., 1993). Other basic amino acids as histidine and lysine, may contribute to DNA binding, so do some polar amino acids as glutamine, tyrosine and aspargine (Krishnan et al., 1996; Peeva and Diamond, 2004; Radic and Weigert, 1994).

The specificity of antibodies is also associated with the binding energy. Specificity of antibodies is determined by its relative affinity. Antibodies with very high affinity to target a specific epitope can bind to similar, but different, epitopes with lower affinity. Dual specificity, cross reactivity, is a common property of anti-dsDNA antibodies (Peeva and Diamond, 2004), and nephritogenic anti-DNA antibodies possess a second specificity for non-nuclear antigens, often against inherent kidney antigens.

Candidates for cross-reaction of anti-dsDNA antibodies are matrix components and cell membrane components. Anti-DNA antibodies are shown to have polyreactivity against extracellular matrix components as heparan sulphate, laminin and α-actinin, all constituents of basement membranes. These molecules are rich in repeating, negatively charged units suitable for binding the cationic residues of anti-dsDNA antibody, (Sabbaga et al., 1989; Amital et al., 2005; Suzuki et al., 1993).

Phospholipids are found in all cell membranes and are exposed in apoptotic cells and in complex with the serum protein β2GP1 shown to be recognized by autoantibodies (Cocca et al., 2001). Anti-dsDNA antibodies are also found to cross-react with structural similarities between phosphodiester groups common to both DNA and phospholipids (Lafer et al., 1981) as well as binding to mesangial cell membranes (Raz et al., 1993) and α-actinin (Deocharan et al., 2002; Mostoslavsky et al., 2001).

1.10 Mechanisms for immune complex deposition in kidneys

In patients with SLE, deposits of immune complex have been identified in several organs, but only in the glomeruli and capillary membranes specific antibodies have been identified. Three mechanisms have been proposed to explain the deposition of anti-dsDNA antibodies in the kidney (Rekvig et al., 2004; Peeva and Diamond, 2004).

One model is based on the possibility that sub-populations of anti-dsDNA antibodies possess a second specificity for non-nucleosomal kidney antigens (Figure1.9A). Nephritogenic anti-dsDNA antibodies are highly cross-reactive and may react with renal tissue antigens that share similar epitopes with DNA. Several reports indicate that α-actinin (Mostoslavsky et al., 2001; Deocharan et al., 2002) laminin (Sabbaga et al., 1989; Amital et al., 2005) and heparan sulphate (Faaber et al., 1986; Rops et al., 2004) or cell membranes may be candidates for antibody recognition (Raz et al., 1993; D'Andrea et al., 1996)

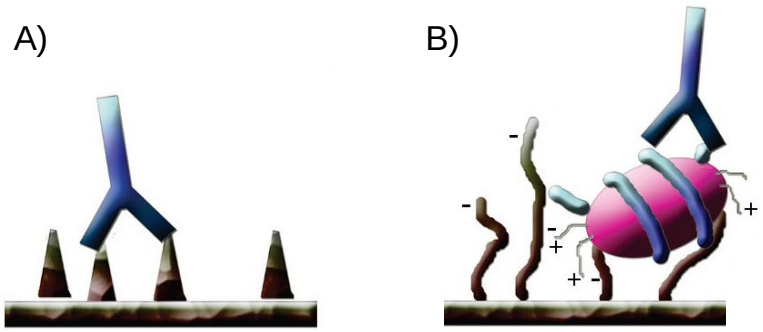

Figure 1.9. Schematic representation of the two hypotheses for the glomerular binding of autoantibodies in lupus nephritis. Direct binding of cross reactive autoantibodies to non-nucleosomal glomerular antigens (A) and nucleosome mediated binding of complexed auto-dsDNA antibody to GBM (B) (Figure modified from (van Bavel et al., 2007)).

The second model suggests deposit of immune complex, and is based on the ability of chromatin to bind to GBM. Via the exposed cationic histone part of the nucleosome, the immune complex is able to bind to the anionic heparan sulphate of the GBM (Kramers et al., 1994; Termaat et al., 1992). Anti-dsDNA antibodies and nucleosomes form immune complexes, and the nucleosome-part directly bridges the antibody to the glomerular constituents (van Bruggen et al., 1996; Lefkowith and Gilkeson, 1996; Berden et al., 1999; van Bruggen et al., 1997a; Rekvig et al., 2004) (Figure 1.9 B). According to this perception, lupus nephritis would be an immune-complex dependent process. Where these immune complexes are formed is not known. They may originate from anti-dsDNA antibodies binding nucleosomes in circulation (Pisetsky, 2004a) or from local renal cells damaged by disease (Mannik et al., 2003; Makino et al., 2003). In glomerular immune deposits, nucleosomes, nucleosome-specific antibodies and nucleosome/antibody complexes have been identified (Berden et al., 1999; Kalaaji et al., 2007). A variant of this model states that anti-dsDNA antibodies deposit in kidney by binding to nucleosomes trapped in advance in the glomerular basement membranes (Bernstein et al., 1995; Grootscholten et al., 2003). DNA and nucleosomes are shown to have affinity for GBM (Mjelle et al., 2007; Izui et al., 1976) There are experimental data and theoretical considerations arguing for and against these nephritic models, but none are proven valid beyond any doubt.

1.11 Hypothesis of the pathophysiology of lupus nephritis

Figure 1.10 brings together elements which are considered important for the pathophysiology of lupus nephritis (Tax et al., 1995) and which is described in this chapter. Data either support or refute topics in this hypothesis.

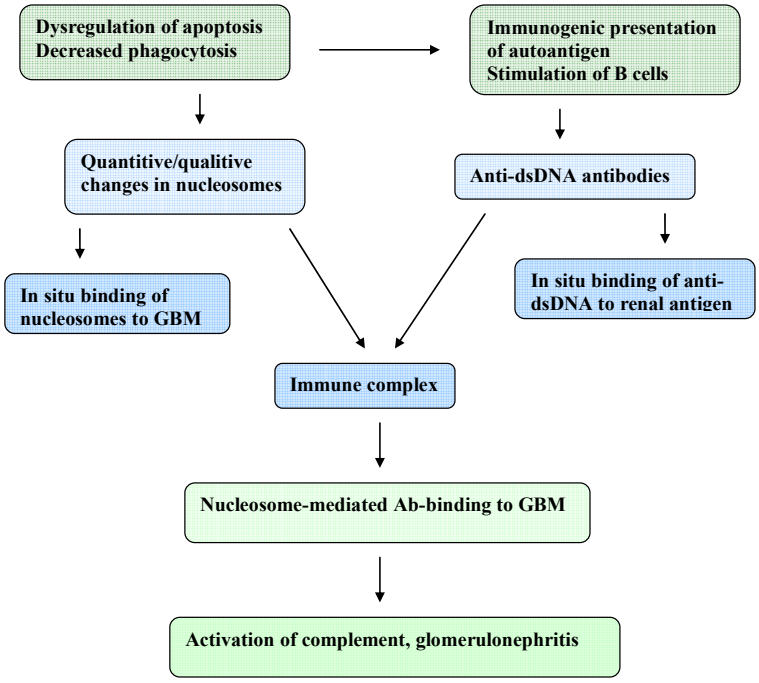

Figure 1.10. Hypothesis for the pathophysiology of lupus nephritis
Dysregulation of apoptosis together with defects in the phagocytosis of apoptotic cells leads to an increased release of nucleosomes. Persisting autoreactive T cells can provide help for the production of anti-dsDNA antibodies. Immune complex is made. Nucleosomes can mediate the binding of the complex to GBM with subsequent induction of glomerular inflammation (Figure modified from (Tax et al., 1995)).

2 AIMS OF THE STUDY

This study is part of the main project of the group, denoted **"**Critical research program on the association between anti-dsDNA antibodies and Systemic lupus erythematosus nephritis".

Anti-dsDNA antibodies are included as a classification criterion for systemic lupus erythematosus, but need a better definition. Sub-populations of anti-dsDNA antibodies have potential to induce nephritis, but there is no known marker to identify pathogenic / non-pathogenic antibodies involved in nephritis (Waldman and Madaio, 2005). Analysis of glomerular target structures for nephritic anti-dsDNA antibodies may reveal whether antibody affinity for DNA, specificity for inherent renal molecules, or availability of target antigens is crucial parameters for developing lupus nephritis (Berden et al., 1999; Rekvig and Nossent, 2003; Rekvig et al., 2004);. There are experimental data and theoretical considerations arguing for and against several of the currently investigated nephritis models, while conclusive information has been missed till now. In order to contribute to the present knowledge of anti-dsDNA antibodies and nephritis, the following aims were defined:

- Analyse the characteristics of antibody that bind, respectively do not bind in vivo in the glomeruli.
- Determine whether the antibodies, when injected into healthy mice, recognize DNA, or cross-reacting, obligate glomerular structure.

3 MATERIALS AND METHODS.

Production of monoclonal antibodies (mAbs) using hybridoma technology has allowed the analysis of individual antibody molecules. The initial production of mAbs was followed by purification, and before injection into healthy mice, the mAbs were tested for quality and quantity. After sampling, anti-dsDNA antibodies were traced in serum and kidney tissue. The design of this study is summarized in Figure 3.1.

Buffers, solutions and media are described in the text as they appear for the first time, and the most important reagents, equipment and instruments used in this study are listed in the appendix.

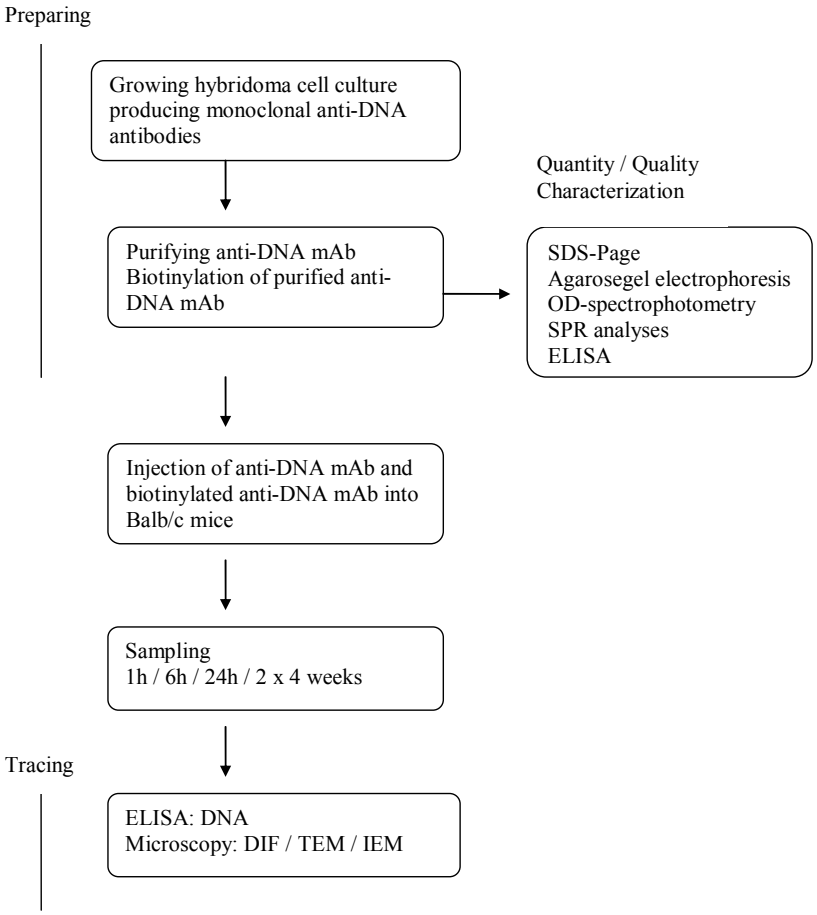

Figure 3.1. Flowchart showing design and methods of this study.

3.1 Hybridoma cell lines

A hybridoma cell line is made by fusion of B cells from spleen of autoimmune mice with an immortal mouse myeloma cell line, for the purpose to produce monoclonal antibodies. The B cells provide the specific antibody and the myeloma cell partner provides the ability to grow indefinitely in culture and allowing antibodies to be secreted continuously. Each hybridoma is a clone with part of a single B cell and all the antibody molecules it produces are identical in antigen-binding site, V-region structure and isotype. These antibodies are called monoclonal antibodies. Nine hybridoma cell lines derived from autoimmune (NZBxNZW)F1 (from here NZB/W) mice were provided by Dr. Tony Marion, Memphis, USA. All antibodies were specific for DNA and were selected on their ability to bind or not bind to nucleosomes and/ or matrigel. Each hybridoma cell culture was coded by Dr. Marion (mAb A-I).

Cell culture

After careful thawing of the cells, they were washed by drop wise applying media (RPMI 1640 + antibiotics / 10% FBS / 2% L-glutamine / Interleukin-6 2 ng/ml) about 10 times the volume of the cells. After centrifugation at 1400 rpm for 5 minutes, the supernatants were removed. The pellets were resuspended in 7.5 ml medium and transferred into T25 ($25cm^2$) culture flasks and incubated at 37^oC in a 5% CO_2 humidified incubator. The procedure was performed in a vertical laminar flow cabinet by standard sterile techniques.

CELLine 1000

The cells were grown until they reached a number of approximately 2×10^6 / ml in 15 ml (total 3×10^7 cells). By ELISA assay, the supernatants were tested for presence of antibodies. If positive, the cells were inoculated into a multi-chamber cell cultivation system that resembles an ordinary culture flask, but is based on membrane technology. This CELLine 1000 from BD Biosciences are especially suitable for antibody production (Figure 3.2)

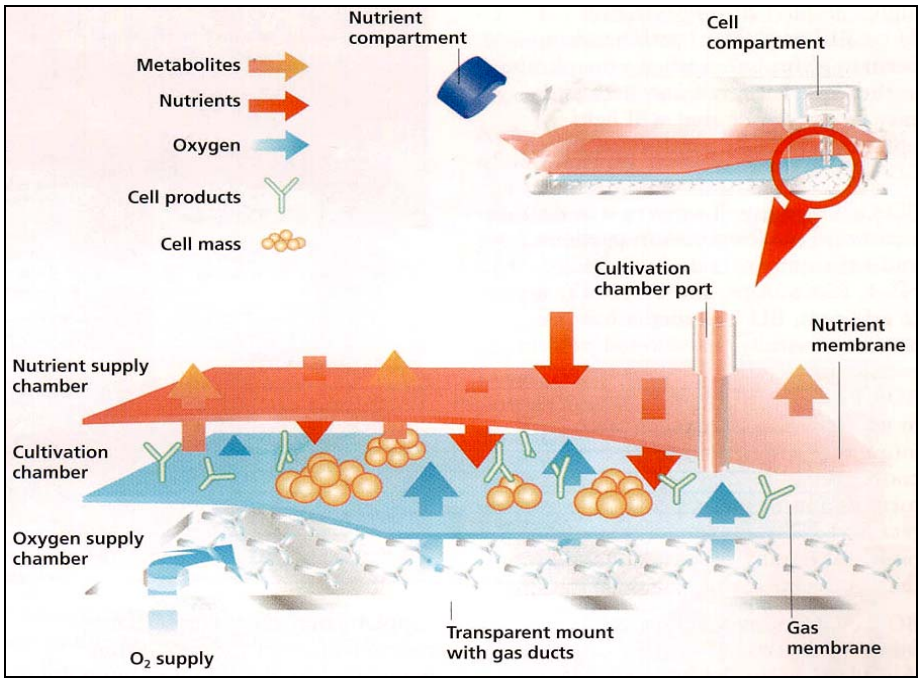

Figure 3.2. CELLine 1000 are suitable for culturing antibody- producing hybridoma cells.
Cells are maintained in a 15 ml cultivation chamber that is separated by a semi-permeable membrane from a one litre nutrient supply compartment. Nutrients and other small molecules can pass across the semi-permeable membrane into the cell cultivation chamber. Cell-secreted products with a molecular weight greater than 10,000 Dalton are retained in the cell growth chamber of the device. A moulded silicone membrane on the bottom of the device allows oxygen to reach the cells from underneath. The cells settle upon the silicone membrane at the bottom of the cell compartment, which provides direct access to oxygen and carbon dioxide gases that rapidly diffuse across the membrane. This approach leads to high cell concentrations within the small volume of medium in the cell cultivation chamber. Separate ports provide selective access to the nutrient supply chamber and the cultivation chamber. The cell chamber is accessed via the cultivation chamber port using a serological pipette (BD, CELLine Membrane Technology)

Before seeding the cells, the number of viable cells was determined by hemocytometer counts of cells stained with 0.4% trypan blue. 2 x10^6 cells/ml were resuspended in 15 ml RPMI 1640, including the additional nutrients as previous described and 20% FBS. The serum concentration in the cell compartment is high to make up for osmotic flux from the nutrient compartment. The pre-wet nutrient chamber is filled with 1 litre of RPMI 1640 supplemented with 2% L-glutamine. The CELLine device containing the cells was incubated in a CO_2 incubator at 37^{o}C for 7 days. Because of the small cell compartment, the produced antibodies are in high concentrate in 15-20 ml of medium.

Harvest of the produced antibodies

On day seven, the cells and the antibody-containing medium were collected from the cell compartment using a serological pipette. The sample was centrifuged at 1400 rpm for 5 minutes and the antibody-containing supernatant was collected and stored at -20^{o}C. The cell pellet was resuspended, the living cells counted and reseeded into the flask at 2.0- 5.0 × 10^{6} /ml and cultured for another 7 days. The media in the nutrient chamber was restored after the third antibody harvest (day 21).

3.2 Purification of monoclonal antibodies (mAb)

Purification of antibodies was performed by affinity chromatography. This technique exploits the strong binding of a specific antibody to an antigen coupled to a solid matrix. Antigen is covalently bound to small, chemically reactive beads, which are loaded into a column. A liquid containing many different molecules, including the one of interest, is passed through the column. The specific antibody bind, the rest is washed away. The bound antibody is then eluted by altering the pH.

Purification based on affinity chromatography

The method was performed by using Protein A-Sepharose 4B, Fast Flow beads from Sigma. These are agarose beads coupled with Protein A, the immunoglobulin-binding fragment from the bacterium Staphylococcus aureus that binds to the Fc region of IgG of mammalian species. The binding capacity is approximately 35 mg/ml determined using human IgG. This resin shows high affinity for mouse IgG subclass 2 antibodies and low affinity for bovine IgG.

The purification of mAbs is important to do since nucleosomes always are released into the culture medium resulting in complex formation of antibodies and nucleosomes. We purified antibodies according to the protocol 3 described by (Kramers et al., 1994). This protocol imply high salt wash after binding of the antibodies to Protein A-sepharose in order to dissociate histones from DNA bound to immobilized mAbs. Then histone-free DNA is completely degraded by DNaseI. After washing the column, antibodies are eluted at low pH and are assumed to be free of contaminating protein and DNA.

Pre-treatment of lyophilized Protein A-Sepharose was performed according to the manufacturer manual. One gram powder was swollen in Buffer A (0.02 M NaH_2PO_4 / 0.15 M NaCl, pH 8.0) for 30 minutes, and gave 4 ml of hydrated gel. Approximately 1.5 ml of hydrated gel 1:1 in Buffer A was applied to the 15 ml column and the 3 ml resin was allowed to settle. The column was washed with 10 ml Buffer A two times. All washing is done by adapting the column to a collecting tube, and spinning at 4000 rpm for 5 minutes.

Culture supernatants were applied to the Protein A-Sepharose column, sealed off in both ends and incubated over night at 4°C at rotation. After centrifugation, the supernatant was discarded (aliquot 1). The column was washed with 10 ml high salt solution (3 M NaCl in PBS (8 %NaCl / 0.2% KCl / 1.45% NaH_2PO_4 / 0.24 % KH_2PO_4, pH 7.4)), (aliquot 2), followed by equilibration with Nuclease Digestion Buffer (40 mM Tris / 6 mM $MgCl_2$, pH 7.5). The resin was incubated with 250 U/ml DNase I (Amersham Biosciences) in Nuclease Digestion Buffer at 37°C at rotation for 1 hour, and the supernatant was discarded (aliquot 3). Finally the resin was washed with Buffer A (aliquot 4), and the antibodies bound to the Protein A was eluted with a solution of 0.1 M citric acid pH 3.0. The column was centrifuged at 4000 rpm for 5 minutes at 4°C, and the supernatant was collected and immediately neutralized by adding 0.5 M Tris (aliquot 5) (See appendix for details). The columns containing the sepharose resin were reused for each supernatant. They were washed with 0.1 M citric acid, pH 2.6 and re-equilibrated with Buffer A. Then the bottom was sealed off, and Buffer A / 20% ethanol were added for storage at 4°C.

We also included commercially purchased polyclonal mouse IgG from non-autoimmune mice (Invitrogen) in the study. This irrelevant IgG (mIgG) was treated according to the same purification procedure as the mAb produced in the laboratory, and was used as negative control.

Dialysis

To prepare the antibodies for injection into mice, the buffer must be exchanged into physical conditions. We used Slide-A-Lyzer dialysis cassettes (Pierce) that hold a sample volume of 3-12 ml. The pore size is 10.000 MWCO (10 kDa), and allow the small molecules of the buffer component to move freely across the membrane until equilibrium is achieved. The antibodies are 150 kDa and remain inside the dialysis chamber. The dialysis cassette was adapted to a buoy floating in a beaker containing 2 litre PBS and adjusted to continuous magnetic stirring.

The PBS was changed several times: First at room temperature for 2 hours two times each, and then 4^{o}C over night, and finally at room temperature for 4 hours.

Concentration of pure mAbs

Before injecting the antibodies into the mice, they needed to be concentrated to keep the injection volume low. To remove liquid from the dialyzed antibodies, we used dialysis tubes of 12-14 kDa (Visking). To remove glycerine, which acts as a humectant to prevent brittleness, the tubes were treated with 1mM EDTA in 2% $NaHCO_3$, 80^{o}C for 30 minutes. Then the tubes were rinsed for 20 minutes in continuously flowing MilliQ water.

The concentration was measured and the volume estimated before the dialysates were transferred to the dialysis tube and buried into Polyethylene glycol (PEG) 35000. According to the acquisition $C_1V_1=C_2V_2$, it was estimated how much liquid to be removed. This is a crude method, and exact adjustments were done by dilutions prior to injection.

Biotinylation

One antibody (mAb G) was biotinylated prior to a bolus injection in nephritic NZB/W mouse to target the exact deposit pattern for this bolus injected antibody. The antibodies were biotinylated using No-weight^{TM} Sulfo-NHS-LC-biotin (Pierce) according to the manufacturer. Briefly, 1ml of antibody solution (2.0 mg/ml) were incubated with 26.6 µl biotin (10 mM) for two hours on ice to yield a 20-fold molar excess of biotin reagent. The excess biotin were removed by centrifugation through a desalt column (Pierce). Proper biotinylation of anti-dsDNA antibodies were analyzed with anti-biotin antibodies (Roche) in an anti-DNA ELISA as described below.

3.3 Quality and quantity of the purified monoclonal antibodies.

Before injecting the antibodies into mice, the final products were tested for purity and concentration, and some chosen characteristics of the monoclonal antibodies were analysed with respect to deposit patterns in kidneys in later investigations.

Quantitation

The concentration of total IgG was determined spectrophotometrically by measuring absorbance at optical density (OD) at wavelength 280nm (1 OD_{280nm} = 0.75 mg/ml antibody). The measurement was performed with ND-1000 Spectrophotometer (Nanodrop Technologies Inc.). IgG reference was by the manufacturer stated as unknown (sample) protein concentrations calculated using the mass extinction coefficient of 13.7 at 280nm for a 1% (10mg/ml) IgG solution.

Agarose gel electrophoresis

Agarose gel electrophoresis is a method to separate DNA molecules due to size and conformation. Our purpose for running this test was to visualize any contamination of DNA in the purified antibody suspension. The limit of detection when using ethidium bromide as intercalating dye is 10-40 ng of DNA. Maximal volume of the samples (20 µl + 5 µl loading buffer (0, 25% bromophenol blue (Baker), 0, 25% xylene cyanol FF (Sigma), 30% glycerol in water)) was applied to 1% agarose gel in TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA) and run for 90 minutes at 90 V. We included the molecular weight standard TriDye1 kb DNA ladder for band size estimation. The DNA bands were visualized in UV-light and documented by GelDoc 2000 (BioRad).

Sodium dodecyl sulphate- polyacrylamide gel electrophoresis (SDS-PAGE)

This is a common technique to separate proteins due to their size as they migrate through an electrical field. SDS is an anionic detergent, which denatures and binds proteins and gives the protein a net negative charge. The reducing reagent, dithiothreitol (DTT), denatures the protein by breaking disulphuric bridges and the effect of different shapes is eliminated. The protein migration rate reflects the mass or the length of the polypeptide chain.

In this study, SDS-PAGE was used to demonstrate protein bands of size corresponding to the heavy and light chain of the IgG antibody molecule. We also looked for possible contaminants and estimated the efficiency of the purification method.

The method was performed according to the manufacturer's manual (NuPAGE Technical Guide, Invitrogen). In short, the samples were mixed with loading buffer and DTT;

heat denatured for 10 minutes at 90^{o}C and applied to a 4-12% Bis-Tris gel. Prestained SeeBlue Plus2 (Invitrogen) was used as molecule weight standard to estimate the band size. The electrophoresis was run at 200V for 40 minutes in an Xcell SureLock Mini-cell filled with MES buffer (50 mM Tris base, 50 mM 3–(N–Morpholino) propanesulfonic acid, 1 mM EDTA, and 0.01% SDS at pH 7.3). The bands were visualized using Coomassie SimplyBlue SafeStain and 20% NaCl to gain additional contrasts, and photographed with GelDoc 2000.

Enzyme –linked immunosorbent assay (ELISA)

An immunoassay is based upon the interaction between an antigen and the corresponding antibody to decide the presence of one or the other. An antigen is attached to the wells of a multiwell plate, and sera, medium or other liquids with the suspected antibody are applied. The antigen will stably bind the antibody allowing unbound antibody to be removed by thorough washing. A secondary antibody conjugated with an enzyme is applied. When a colourless substrate is added, it is converted into a coloured precipitate proportionally to the amount of enzyme present. The intensity of this colour can be photometrical measured at 492nm. This is a sensitive way to tell how much antibody is present; there is a metric relationship between bound antibody and colour intensity within certain limits.

In this study, solid phase ELISA were performed to characterize the pure mAbs ability for binding to dsDNA and possible cross-reacting antigens. The performance was based on methods in the given references. We also estimated the kinetics of removal of the injected anti-dsDNA antibodies from the circulation in mice, by ELISA.

Coating with antigens

The polystyrene microtiter plates (MaxiSorp, Nunc) were coated with different antigens. These antigens were selected based on the suspected ability to bind anti-dsDNA antibodies.

Calf thymus dsDNA (dsDNA) was purchased from Sigma and diluted to 10 µg/ml in PBS, and 50 µl was added to each well, sealed with plastic cover and incubated at 37^{o}C over night (Kalaaji et al., 2006b; Rekvig et al., 1997b).

Nucleosomes extracted from A431 cell (Andreassen et al., 1999a) were sonicated on ice at 40% effect, 1 pulse of 10 seconds (Sonifier), diluted to 10µg /ml in dH_2O and 50 µl was applied to the wells. The plates were sealed and incubated at 37^{o}C over night.

Phosphatidylserine (PS) from Sigma was diluted to 10ug/ml in 96% ethanol. 50 µl was added to each well and incubated in 37°C for 2 hours with no lid (Matsuda et al., 2002; Hasselaar et al., 1990; Cocca et al., 2001). Additionally we included 10 µg/ml β2GP1 (Crystal Chem) in complex with PS and incubated as above.

Phosphatidylcholine (PC) from Fluka was coated as described for phosphatidylserine.

Matrigel matrix was purchased from BD Biosciences and treated with DNase I (Amersham) according to the manufacturer and followed by dilution to 10µg/ml in PBS. 100 µl was applied to each well, sealed and incubated over night at room temperature. Following washing by PBS / 0.1 % Tween20, the wells were blocked with 150 µl 10mM Tris-HCl, pH 7.4 / 5% BSA for 1 hour in 37°C (van Bruggen et al., 1997b; Mohan et al., 1999).

Dilution of primary antibodies applied to ELISA

The samples were diluted in different solutions according to the assay; for dsDNA and nucleosome the samples were diluted in RPMI enriched with 10% FBS and 2% L-glutamine, for PS and PC the samples were diluted in PBS and for matrigel the samples was diluted in PBS / 1% BSA. (See degree of dilution as indicated on respective figures of the results.)

Relevant control antibodies were included in each ELISA for intra-assay validation of results; for dsDNA and nucleosome assays the murine anti-dsDNA monoclonal antibody 163p77 were used, for PS og PC anti-phosphatidylserine clone 1H6 (Upstate) and for matrigel, anti-laminin antibody (Sigma).

The plates were sealed with plastic cover, and incubated in 37°C for 1 hour. Washing was preformed by Wellwash 4 (Labsystems) with the respective buffers; PBST (PBS / 0.02% Tween 20) for dsDNA and nucleosomes, PBS / 0.5% gelatine for PC and PC and PBS / 0.1% Tween20 for matrigel.

Secondary antibodies

Anti-mouse IgG and anti-rabbit IgG conjugated with the enzyme horseradish peroxidase (Sigma and DAKO respectively) was diluted 1:2000 in the respective solutions, added to the washed wells, sealed, and incubated for 1 hour at 37°C. Washing was performed as above.

Substrate

Substrate was made of 12 ml phosphate-citric-buffer (0,2M Na_2HPO_4 / 0,1M $C_6H_8O_7$ pH 5.0/ 8 mg O-phenylenediamine dihydrochloride / 10 µl hydrogenperoxide. An amount of 50 µl was added to each well, and incubated in room temperature until brownish colour develops in the positive control (about 10 min). The reaction was stopped by adding 25 µl 3M HCl. Finally, the measurement of the degree of substrate conversion was done spectrophotometric at 492 nm (Multiscan Ascent, Thermo)

3.4 Surface Plasmon Resonance (SPR) analyses

SPR is a technology that enables the real-time measurement of interaction between two molecules. As molecule complexes form and break at a sensor chip, the refractive index change and an optical SPR response is observed as a reduction of reflected light (Figure 3.3). This is proportional to the changing mass of material, and is characterized in terms of association and dissociation. Affinity is the strength of the interaction between the reactants, and define how much complex is created at equilibrium i.e. balance between association and dissociation. Kinetics describes the speed of the on-off interaction

The Biacore system of SPR is an integrated technology where the sensor chip, flow system and SPR detection unit work together. The micro flow system delivers the samples to the sensor chip surface with a constant flow and concentration. The analyte in the sample associates with the immobilized ligand (Figure 3.4) on the surface, the mass on the surface changes and a response is recorded. After sample injection, buffer flow allows dissociation of analyte from ligand. Regeneration and stabilizing is done to bring the surface back to basic before next cycle. The SPR is visualized in a sensorgram showing response units (RU) / time (Figure.3.5)

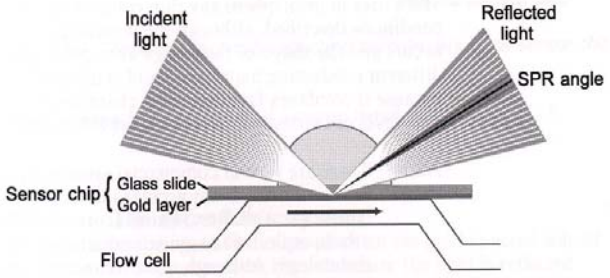

Figure 3.3. The SPR detection system

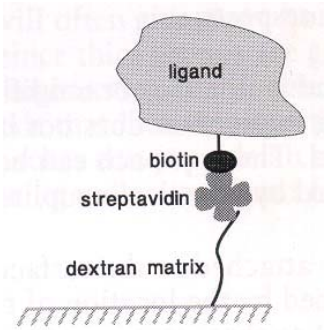

Figure 3.4. Biotinylated ligand captured streptavidin on chip surface

In this study we measured the affinity of purified anti-dsDNA antibodies for immobilized DNA. Mouse genomic DNA (Calbiochem, Germany) was biotinylated using a biotin-16-dUTP (Roche, Switzerland) and Nick translation kit (Vysis, Abbott Molecular Inc, IL, USA) as described in the protocol from the manufacturer. For short; DNA was labelled in 50 µl reactions containing 1 µg genomic DNA, 2,5 µl 0,2 mM biotin-16-dUTP, 5 µl 0,1 mM dTTP, 10 µl dNTP mix, 5 µl 10x translation buffer, and 10 µl nick translation enzyme. Reactions were incubated at 15^{o}C for 8 hours. Biotinylated DNA was coupled to streptavidin coated sensor chips (SA series S) (Figure 3.4) to an immobilization level of approximately 700 RU (Mjelle et al 2008, submitted).

SPR analyses were performed using the Biacore T100 system manufactured by Biacore, Sweden. The protocol was performed according to suggestions in the manufacturer manual, and all necessary buffers, solutions, chips and tubes were obtained from the company. Anti-dsDNA antibodies were diluted 2-folds in running buffer from 500nM to 0.488nM and injected over the chip surface with a flow rate of 30µl/min for 6 minutes followed by injection of running buffer for 10 minutes. Regeneration was performed with 50 mM NaOH at 30µl/min for 2 minutes. After stabilizing the chip with a normalizing buffer for 2 minutes, the next cycle started. The sensograms were analysed by the Biacore T100 control software and evaluation software. The kinetic of the interaction, i.e. the rates of association (k_a) and dissociation (k_d), was determined from the information in the sensogram (Figure.3.5), and the affinity (K_D) was calculated from the ratio between k_a and k_d, using the Langmuir model for 1:1 interaction.

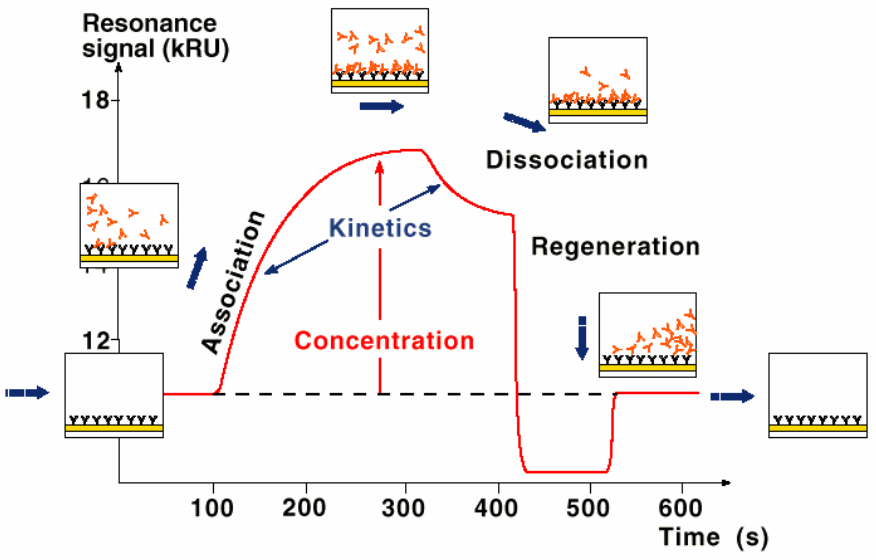

Figure 3.5. A typical sensogram for surface plasmon resonance. Equilibrium dissociations constant k_D (affinity) was calculated from the ratio between complex formation, association ($k_{a)}$, and the rate of complex decay, dissociation (k_d). $k_D = k_d / k_a = [A] \times [B] / [AB]$, where A is the analyte in solution and B is the ligand on surface.

3.5 Injection of monoclonal anti-dsDNA antibodies into mice

Mice

BALB/c, NZB and NZW mice were purchased from Scanbur (Sweden) and Harlan (Oxon, UK), and were housed in the facilities of the Animal Research Department at the Institute of Medical Biology, University of Tromsø. Treatment and care of the animals were in accordance to with the guidelines of Norwegian Ethical and Welfare Board for Research Animals and the study was approved by the Institutional Review Board. Female BALB/c mice were injected at a young age, approximately 10 weeks old. Hybrid lupus-prone NZB/W mice were generated by crossing the NZB and NZW mouse strains. Totally in the study we used 15 injected BALB/c mice, 1 non-injected BALB/C mouse, 1 injected NZB/W and 1 non-injected NZB/W mouse as specified below.

Injection design

In this study, four of the nine available mAbs were chosen for injection. The antibodies randomly chosen were produced by cell line B, C, E and G. We also included a biotinylated antibody from cell line G and irrelevant mouse IgG.

Each type of pure mAb and irrelevant mouse IgG was injected into three parallel healthy BALB/c mice, totally 15 mice. Mouse 1 and 2 received a single bolus injection of 0.5 mg antibody and were sacrificed after respectively 6 and 24 hours. Blood samples were collected at time points 1, 6 and 24 hours for later tracing of antibodies in serum, and urine was tested for proteinuria. Mouse 3 got repeatedly injections of 0.2 mg twice a week for 4 weeks. Prior to each injection, blood sample was collected and proteinuria measured (Table 3.1). One nephritic NZB/W mouse got one bolus injection of 0.5 mg biotinylated mAbG and was sacrificed after 24 hours.

Table 3.1. Injection of purified antibodies. Each antibody was injected into 3 mice and sacrificed after 6 hours, 24 hours and 4 weeks. Blood and urine was collected prior to injection and at different time intervals.

Serial samples of serum and urine					
	0 hour		1 hour	6 hours	24 hours
	Sample	Injection	Sample		
Mouse 1	Blood/urine	0,5 mg	Blood/urine	Blood/urine Sacrifice	
Mouse 2	Blood/urine	0,5 mg	Blood/urine	Blood/urine	Blood/urine Sacrifice
Mouse 3	Blood/urine	0,2 mg Twice a week (x8)	Blood/urine x8 Sacrifice		

3.6 Sampling blood, urine and tissue from sacrificed mice

Figure 3.6. Collecting blood and organs from mice sacrificed at the Animal Research Department, University of Tromsø.

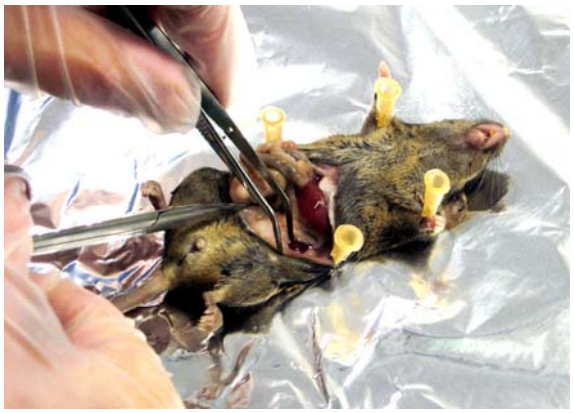

Figure 3.7. NZB/W mouse having the kidneys removed for further studies

Blood: Samples were collected from the mice and transferred into a micro tube. The blood was centrifuged at 2000 rpm for 5 minutes at 4^{o}C and serum was transferred to new tubes and stored at -20^{o}C until ELISA-testing.

Urine: Examination of urine is an important method to detect and monitor kidney dysfunction, and is criteria no.7 in the ACR classification of SLE. When handling the mice, they spontaneously urinate, and the urine spots were immediately tested for proteinuria by urine dipsticks from Bayer Diagnostics. Values of 1+ are approximately ≤ 0.3 g/L protein in urine and this is regarded as normal condition. Further, 2+: 0.3-1 g/L; 3+:1-3 g/L and 4+, ≥ 20g/L.

Kidney: Kidneys from the mice were sampled for examination by different types of microscopy. The main issue is to preserve the ultra structures and the native folded protein to whom the detecting antibodies might bind, and leaving no artefacts.

For direct immunofluorescence microscopy, about one quarter of a kidney was placed in a plastic mould and embedded in OCT compound (TissueTek, Sakura). Isopentan in a metal beaker was precooled in liquid nitrogen and the plastic mould with its content was, with the help of a forceps, held into this solution, until it progressively froze from outside and in. Then the plastic mould containing the frozen piece of kidney was put in liquid nitrogen, and later stored at -70^{o}C. For transmission electron microscopy and immune electron microscopy, a slice from the cortex area of the kidney was cut into small pieces and chemically fixed in respectively 8% paraformaldehyde and McDowell's fixative (see appendix).

3.7 Tracing of injected anti-dsDNA antibodies: Microscopy and ELISA

To detect the presence of anti-dsDNA antibodies, two methods was used: ELISA assay to analyze serum (described in 2.3.4) and microscopy for examination of kidneys for morphological changes and antibody binding: (TEM, IEM and DIF)

Direct (DIF) and (indirect) immunofluorescence microscopy

To get an impression of the overall deposits of anti-dsDNA antibodies in the glomerulus and for detecting subcellular localization, we performed immunofluorescence microscopy on the kidney sections. We also attempted to detect a possible co-localization of C1q and IgG (Kalaaji et al., 2006a; Kramers et al., 1994)

In this study the sections were stained to trace IgG deposits and C1q. Sections from NZB/W mouse and non-injected BALB/c mouse represented positive and negative controls. The fluorophore-labeled antibodies are emitting green and red colour in fluorescent microscope at respective wave lengths of 488nm and 568nm.

Cryosections (4μm) of kidneys of injected mice and control mice were transferred to super-frost slides, and allowed to dry in room temperature for 15-20 minutes. Then the sections were incubated with rat anti-mouse C1q (Cellsciences) diluted in PBS with 10% goat serum and 1% BSA for 30 minutes in a moisture chamber at room temperature. Subsequently, the slides were incubated as above with goat anti-mouse IgG conjugated with AlexaFlour568 (Molecular Probes) to detect deposits of IgG autoantibodies and with donkey anti-rat IgG conjugated with Alexa Fluor 488 (Molecular Probes) to detect weather the autoantibodies co-localize with rat anti-mouse C1q. Finally the sections were washed with PBS and sealed under a cover glass. The sections were immediately investigated in fluorescent microscope (BX51, Olympus), and images were processed using Cell ^F soft imaging system from the same manufacturer. (See appendix for detailed procedure).

Electron Microscopy (EM)

The basic principles of electron microscopy are similar to those of light microscopy; the main difference is that electromagnetic lenses, not optical lenses, focus a high-velocity electron

beam instead of visible light. EM can magnify specimens up to 2 million times and give an effective resolution of 0.10nm. The great resolution and magnification is due to the wavelength of an electron that is much smaller than that of a light photon. Our examination of mice kidneys is done at 20-50 000 x magnification.

The technique relies on contrast staining of the biological sample to strengthen the differentiation of the components. Heavy metals are used for this purpose. They appear dark on the micrograph because they diffract most of the incoming electrons. Without staining, the beam of electrons passes thorough the sample uniformly and the sample appears bright and diffuse (Bozzola and Russell, 1992).

Transmission Electron Microscopy (TEM)

This is a method to investigate the topography and morphology of tissue and cells. We were interested in analysing for changes of the morphology in the glomerulus of mice injected with mAb, without detecting the antibodies themselves. The fixed kidney samples was embedded in plastic, contrast stained with osmium, uranyl acetate and lead, and cut into ultra thin sections by staff at the Electron Microscopy Department, University in Tromsø. The sections were examined using a Jeol JEM-1010 transmission electron microscope.

Immune Electron Microscopy (IEM) and Co-localization IEM (CIEM)

Immunohistochemistry for EM was used for ultrastructural detection of glomerular loci for deposits of autoantibodies and their possible co-localization with DNA. This method combines cryoultra-microtomy together with contrast staining and labelling of specific molecules of interest (Tokuyasu, 1986). Colloidal gold are used as electron dense tags, and are conjugated with Protein-A for binding to Fc region of the probing antibody. The gold particles come in different known diameters of 5, 10 and 15 nm for identification and allow co-localization of two different proteins in the same section. Colloidal gold do not appear naturally in biological material.

To perform this experiment, fixed kidneys from sacrificed mice were infused with 2.3 M sucrose and frozen on specimen pins in liquid nitrogen. Ultrathin cryosections of 80nm were cut with an ultramicrotome and mounted on formvar-coated specimen grids. This was done by staff at the Electron Microscopy Department.

The co-localization IEM is based on double-labeling assays. Presence of glomerular in vivo bound antibodies were demonstrated by incubating the sections with rabbit anti-mouse IgG antibodies (ICN/Chappel) followed by protein A conjugated with 5 nm gold particles (PAG-5) (University of Utrecht, The Netherlands). The subsequent procedure was initiated by separate incubations with glutaraldehyde to block for unspecific epitopes and glycine to saturate free aldehyde groups. To colocalize dsDNA, the sections were incubated with experimental murine mAbs (anti-dsDNA 163p77) followed by incubation with rabbit anti-mouse IgG antibodies and subsequently with PAG-10 (10nm gold) to visualize the presence of the last added antibodies. Injected biotinylated mAb G was detected by rabbit anti-biotin antibody followed by PAG-10. Finally the grids were treated with methylcellulose and uranylacetate and left to dry in room temperature. The antibodies were diluted in PBS/1% fish skin gelatine (Sigma-Aldrich) to block for nonspecific binding of Abs, and each step was followed by washing in PBS (Webster and Webster, 2008)

Specificity and validity of double-labeling of autoantibodies and experimental antibodies for co-localization was verified using irrelevant antibodies like anti-biotin or anti-FITC antibodies instead of the anti-dsDNA mAb 163p77. In that situation, no 10 nm gold particle appeared in the sections. The sections were examined with transmission electron microscope (Jeol 1010), and micrographs were taken at 25-50k total magnification (see appendix for detailed procedure).

4 Results

4.1 Purification of monoclonal anti-dsDNA antibodies

The four hybridoma cell lines included in this experiment were monitored for antibody production every week and shown to have greatest antibody production the first week, decreasing the next three weeks (data not shown). This was consistent with the count of viable cells determined by staining with tryptophan blue exclusion test. The purification protocol is performed on the first and second harvest for all cell lines.

The purification method is based upon affinity chromatography using Protein A-Sepharose beads. During the performance of the procedure, samples were taken after each significant step to analyze it according to loss and purity. This was especially important during the first performances, when we were optimizing the method. (Kramers et al., 1994).

The efficiency of the purification protocol is shown with four different methods: OD-spectrometry, ELISA, SDS-PAGE and agarose gel electrophoresis, all showing consistent results. The final product of all cell lines indicates satisfactory amount and purity of mAbs to be used in this study (see below).

Protein concentration at stages in the purification protocol

The protein concentrations was measured by Nanodrop and presented in Table 4.1. The samples collected after Protein A-Sepharose treatment contains a load of proteins. These are serum proteins contained in the medium and waste product from the hybridoma cells. Samples from the other steps show low IgG, indicating that mAb are still hanging on to the beads, and the eluation with citric acid at pH 3 leads to release of the purified antibodies (aliquot 5). After dialysis, aggregates were seen for all antibodies, except mAb G. There still may be aggregates present, but too small to be seen. The dialysates was centrifuged at 2500 rpm, 5 min at 4^{o}C. Supernatants were transformed to a new tube and aggregates were resuspended in 5 ml PBS. Both were measured at A280nm. For injections and characterisations the supernatants were used, and the aggregates were discharged. The amount of commercial mouse IgG purchased from Invitrogen was 15 mg at start and the final outcome was 8.255 mg, showing that 50% was lost during the purification procedure.

Table 4.1: Protein concentration (mg/ml) measured at different steps in the purification of the antibodies.

	Protein concentration (mg/ml)									Total outcome (mg)†	
	Aliquots 1-5										
mAb	*After sepharose	*After saltwash	*After DNase	*After 1.wash	Eluate + Tris	Dialysat, Supernat.	Dialysat, pellet	Rinsed cassette	PBS	Supernat.	Pellet
C	2.33	0.18	0,13	0,00	0,76	0,26	0,36	0,05	0,00	6.8	4.2
B	2.26	0,28	0,06	0,22	0,41	0,36	0,75	0,15	-0,00	10.0	7.0
E	2.55	0,40	0,35	0,07	1.10	0,39	1.49	0,02	0,03	12.0	10.5
G	3.13	0,15	-0,02	-0,06	0,51	0,32	-	-0,01	-0,05	8.5	-

* Flow through.
† Total outcome found by multiplying concentration and volume.

ELISA to analyse for presence of anti-dsDNA antibodies in stages of the purification protocol

To check for presence of anti-dsDNA antibodies in aliquots collected from the different purification steps, microtiter plates were coated with calf thymus DNA and ELISA was performed according to the previously described protocol. Figure 4.1 shows the profile using antibody C as an example. The same profile also applied to mAb B, E and G.

After sepharose purification, some amount of antibodies is detected in the flow trough indicating that not all mAbs bind the Protein A-Sepharose columns. The binding capacity of 35mg/ml is not exceeded, so this may be because the binding was inefficient. A relative high amount is also lost during the 1.washing step after DNaseI treatment, especially when we consider the volume of 10 ml. This may reflect loss of antibodies that might have cross-bound the column through nucleosomes from the cell culture supernatant. During the DNaseI step this binding will be broken and subsequent washing will lead to the release of antibodies. However, the OD measured at this step, shows no loss at the washing step, indicating that the amount of Abs is low, but the activity of the Abs lost is high. The final product shows high values of antibodies, both in pellet and supernatant.

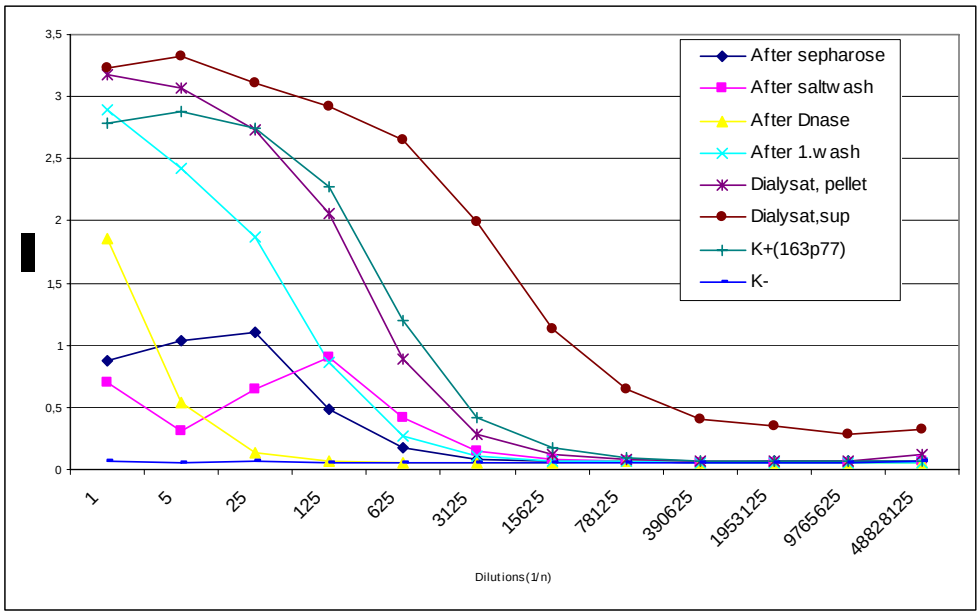

Figure 4.1: Solid phase ELISA: Binding of anti-dsDNA antibodies to dsDNA. Relative amount of anti-dsDNA antibodies measured in different steps (aliquots 1-5) in the purification procedure using antibody C as an example. Fivefold dilution analyses were performed using calf thymus DNA as target antigen in ELISA. The same antibody profile also applied to mAb B, E and G. *Flow through, ¤after purification.

SDS-PAGE to analyse for presence of non-IgG proteins in purified mAbs

SDS- polyacrylamide gel electrophoresis was performed to check for antibodies and possible contaminations of proteins in the different purification steps. The results demonstrated a heavy smear of different types of proteins contained in the hybridoma culture medium in flow through after applied to sepharose column (Figure 4.2, lane 2). Washing with high salt solution purified the mAb more extensively, but some of the mAb were also lost in this treatment (lane 3 and 4). The fragment of >62 kDa in lane 3-6 may be albumin which is the main constituent in serum-enriched medium and have a mass of 69 kDa. After several wash steps and elution of mAbs, the albumin content were removed. The distinct fragments on lane 9-14 correspond to IgG antibody heavy chain (50kDa) and light chain (25kDa), and the intensity of the fragments indicate high concentrations of antibodies in the applied sample. The final products were pure as no other bands were detected (lane 11 and 12).Weak extra fragments (> 62kDa) in lane 9, 10, 13 and 14 indicate minor presence of unknown proteins or aggregates of IgG chains.

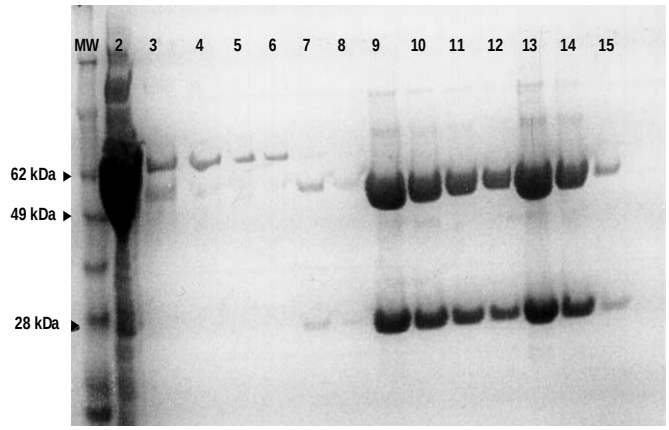

Figure 4.2: SDS- PAGE showing protein content in flow through from different steps (aliquots 1-5) in the purification procedure. Antibody C is used as an example. 12 µl are applied for all samples. Lane 1: Marker (SeeBlue Plus2) Lane 2: Through flow after application on Prot.A-sepharose (column 1) Lane 3-4: After salt wash (column 1 and 3), Lane 5-6: After DNAse, Lane 7-8: After 1.wash, Lane 9-10: Eluat + Tris, Lane 11-12: Dialysat-supernatant 1 and 2, Lane 13-14: Dialysat-pellet, Lane 15: Rinsed dialysis cassette. Maximal volume of the samples (12µl) was applied and the bands were visualized using Coomassie SimplyBlue SafeStain and 20 % NaCl.

The final purified antibodies were tested for presence of protein fragments corresponding to IgG antibody heavy and light chain. This was demonstrated by SDS- PAGE (Figure 4.3).With the SeeBlue Plus2 ladder as a guide; the distinct fragments correspond to heavy chain (50 kDa) and light chain (25 kDa) for antibody E, B and C. Antibody G demonstrated two fragments for the light chain. There is no obvious reason why it appears to be two different 'versions', but may be explained by post synthetic modifications in the light chain.

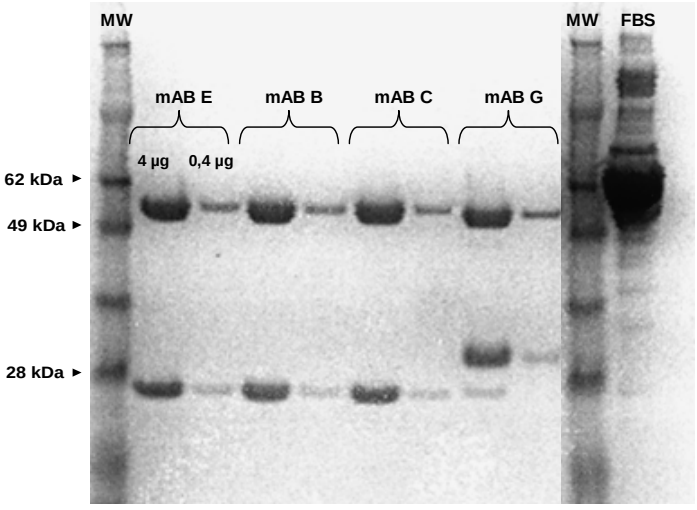

Figure 4.3: SDS-PAGE showing heavy and light chains of pure IgG anti-DNA antibodies. For each antibody, an amount of 4µg and 0.4µg is applied to the gel. MW indicates the molecular weight standard and FBS are foetal bovine serum.

Agarose gel electrophoresis to analyse for presence of DNA in purified mAbs

Agarose gel electrophoresis showed no contamination of DNA in any step of the purification, except for a diffuse smear about 2.0 to 1.0 kb in size in the flow through after sepharose treatment, (Figure 4.4, lane 2) reflecting DNA from dead cells in the cell culture. There was no sign of DNA not before or after DNase treatment, so the need to use that enzyme may be discussed. However, there still might be traces of chromatin present, but below detection level of 10-40 ng/well. Antibody G was used as an example and is representative for the other antibodies. The lack of DNA contamination in the final purified antibodies was verified by nano-scaled electrophoretic assay on Agilent Bioanalyzer (data not shown).

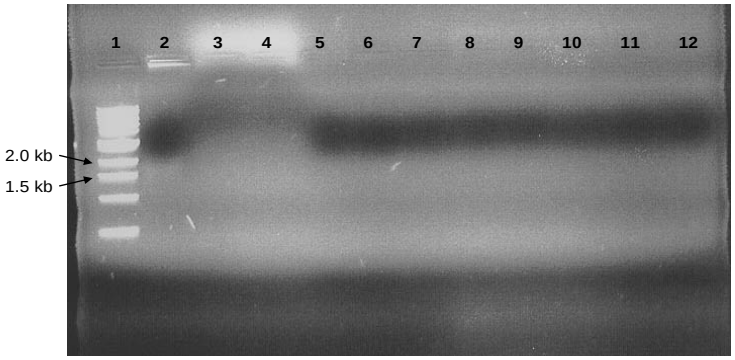

Figure 4.4: Agarose gel electrophoreses to detect DNA in flow through from different steps (aliquots 1-5) in the purification procedure. The cell line producing monoclonal anti-dsDNA antibody G (mAb G) is used as an example and is representative. Lane 1: marker (1 kb TriDye), lane 2: Through flow after application of prot.A-sepharose (column 1), lane 3-4: After salt wash (column 1 and 3), lane 5-6: After DNase, lane 7: After 1.wash, lane 8-9: Eluat + Tris, lane10-11: Dialysate1 and 2, lane 12: Rinsed dialysis cassette. Maximal volume of the samples (20µl) was applied, ethidium bromide used as intercalating dye and DNA is visualized in UV-light.

4.2 Characteristics of purified monoclonal anti-dsDNA antibodies

Characterization by different ELISA assays

The nine hybridoma cell lines A-I, producing antibodies specific for dsDNA, were originally selected according to the antibodies ability to bind or not bind nucleosomes and /or matrigel in ELISA assay. However, for the present study, hybridoma cell lines B, C, E and G producing anti-dsDNA mAbs were randomly selected from the original nine hybridoma cell

lines. Analyses of these antibodies showed, although to different degrees, binding to all ligands. Results are based on spectrophotometric counts visualized in Figure 4.5, and titers are summarized in Table 4.2.

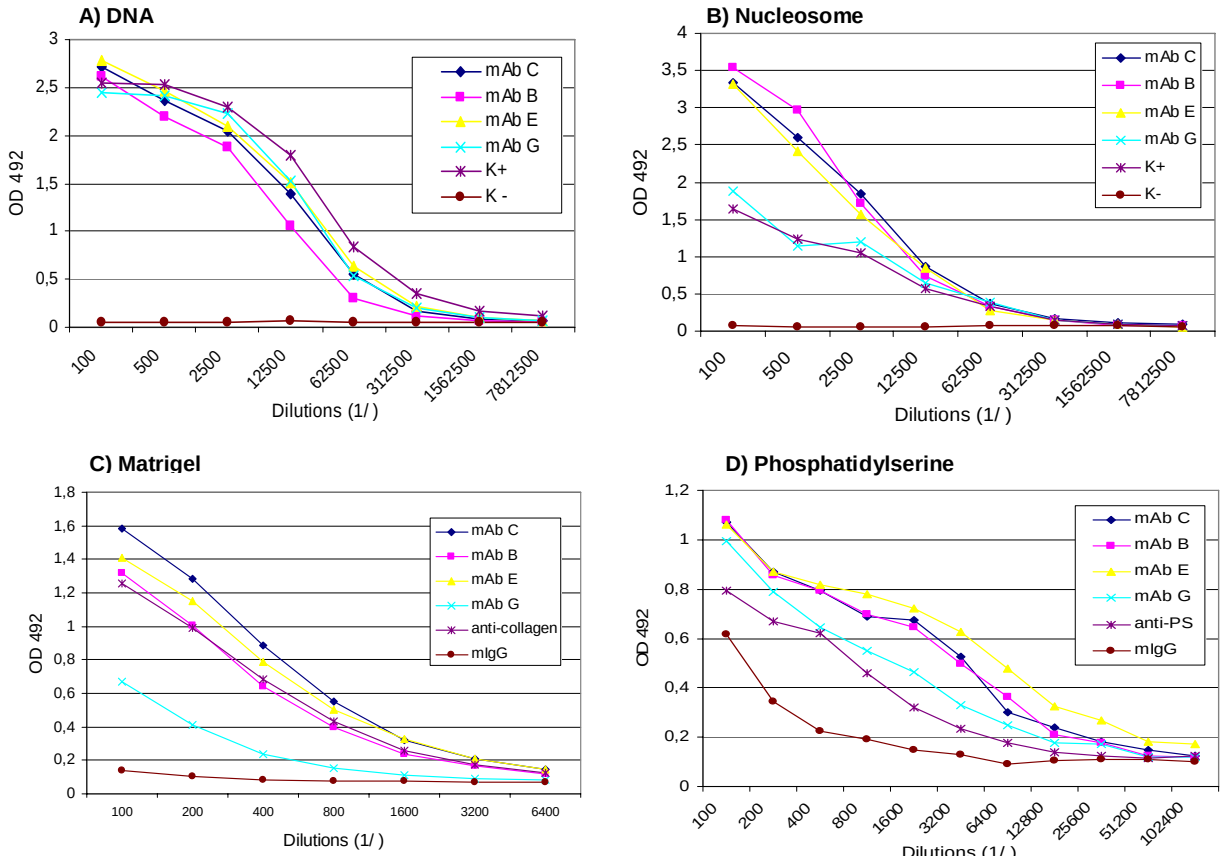

Figure 4.5: Solid phase ELISA showing binding of pure anti-dsDNA antibodies to DNA, nucleosomes, matrigel and phosphatidylserine. The antibodies was diluted fivefold (A and B) or twofold (C and D), starting with 2 mg/ml mAb diluted 1:100 (=20µg).

Calf thymus DNA: As expected, binding of anti-dsDNA antibodies to DNA showed high specificity for all the antibodies (Figure 4.5, A). Mouse IgG was tested and showed no binding.

Nucleosomes: Binding of anti-dsDNA antibodies to nucleosomes showed high reactivity for all antibodies (Figure 4.5, B)

Matrigel matrix is a solubilised basement membrane preparation extracted from the mouse Englebreth-Holm-Swarm tumor. The main components are extracellular matrix proteins as laminin, collagen IV and heparan sulphate proteoglycan and this is used as GBM surrogate. All the antibodies showed binding to matrigel, although mAb G showed significant lower binding capacity than the other (Figure 4.5, C). Irrelevant mouse IgG showed no binding.

Phosphatidylserine (PS) is a phospholipid found in cell membranes and is exposed in apoptotic and necrotic cells. It is also able to bind anti-dsDNA antibodies. All tested antibodies showed specificity for PS. Irrelevant mouse IgG also showed binding, but to a much lower extent (Figure 4.5, D), (Table 4.2). Preliminary tests using RPMI/sera as dilution media showed no binding of mIgG and the positive control was very low, but the samples showed binding much like as the binding values in matrigel (Figure 4.5 C). Addition of β2 glycoprotein I (β2GPI) is reported to enhance binding (Cocca et al., 2001) but we did not find this to affect the result. Similar binding profile was observed for phosphatidylcholine (data not shown).

Table 4.2 Binding capacity of purified mAbs to different antigens

	Antigen (titer)			
mAb	DNA	Nucleosome	PS	matrigel
B	38000	30000	8200	700
C	62500	37500	6400	1100
E	80000	35000	13000	950
G	70000	12500	3200	190
mIgG	÷	nd	250	÷

Titer values were defined as the point where dilution/titration curve are crossing the line for about 50 % of max binding of a reference antibody. The cut-offf value is set at OD^{492} 1 for DNA and nucleosomes, OD^{492} 0, 6 for matrigel and OD^{492} 0, 4 for phosphatidylserine.

Surface Plasmon Resonance for the analyses of affinity

By SPR analyses performed on BiacoreT100 system, we tested the affinity for DNA of each of the individual sub-populations of antibodies. The antibodies were injected at serial dilutions, and analyzed by Biacore T100 evaluation software using the linear Langmuir model of 1:1 binding. The 1:1 model has provided the most reliable results for assessment of antibody-antigen interactions, and informs about relative differences in intrinsic affinity.

The sensorgrams demonstrates the dose-response of antibodies binding to DNA (Figure 4.6). Similar response unit (RU) was obtained for three of the mAbs, while mAb G showed significant lower response units, suggesting that less analyte are bound to the ligand. This indicates that the chip contains less binding sites for antibody G than for the other antibodies. k_D values over 10^{-8} are regarded as high affinity for antibodies, and mAb G and C, showed very high values (k_D 5.814x10^{-11} and k_D 1.870x10-^{11} respectively) (Table 4.3). Irrelevant mouse IgG showed no binding (data not shown)

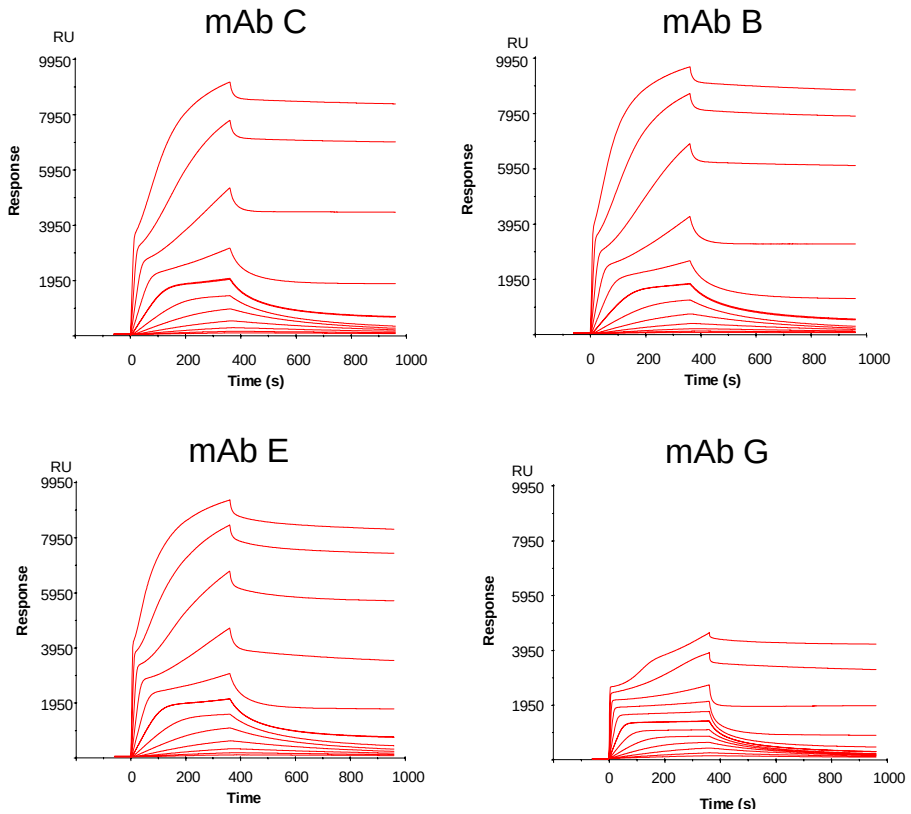

Figure 4.6: **Sensorgrams for the interaction of anti-dsDNA antibody binding to DNA coated chip.** Each antibody was measured at 11 concentrations (twofold dilutions from 500nM to 0.488nM) and analysed with Biacore T100 evaluation software. The response units (RU) reflect the binding of the analyte to the DNA-coated chip in real time measured in seconds (s).

Table 4.3 Anti-dsDNA antibodies affinity for DNA calculated by the Langmuir 1:1 model.

	Rate constant		Affinity constant
mAb	k*a*(1/Ms)	k*d* (1/s)	K_D (M)
C	3.77×10^4	7.04×10^{-7}	1.87×10^{-11}
B	1.70×10^4	1.04×10^{-5}	6.12×10^{-10}
E	1.27×10^4	7.44×10^{-5}	5.85×10^{-9}
G	6.12×10^3	3.56×10^{-7}	5.81×10^{-11}

k_a(1/Ms) = Amount of complex formed per second in a 1 molar solution. k_d(1/s) = The fraction of the complex that decay per second. K_D(M) = Amount of complex formed at equilibrium.

4.3 Proteinuria in experimental mice injected with mAbs

Following bolus injection of antibodies into healthy BALB/c mice, incidentally increased levels of proteins in urine was observed. This was demonstrated by a rise of 1-2 values one hour after injection. Further, the proteinuria level decreased back to normal or stayed the same 6-24 hours after injection. In general, repetitive injections showed the same pattern. There could not be observed any significant differences between the different antibodies. The irrelevant mouse IgG control did not induce proteinuria (see Table 4.4 for details)

Table 4.4 Proteinuria in BALB/c mice injected with purified antibodies.

			Proteinuria measured after											
			Bolus injection				Repetetive injections							
							1	2	3	4	5	6	7	8
Mouse nr	Strain	Ab	0h	1h	6h	24h	0h/1h	0h/1h	0h/1h	0h/1h	0h/1h	0h/1h	0h/1h	0h/1h*
B1	Balb/C	C	+1	+2	nd									
B2	Balb/C	C	+1	+2	+1	+2								
B3	Balb/C	C						+1+2/+3	+1/+2+3	+2/+3	+1/+2	+1/+3+4	+1/+2	+2/+4
B4	Balb/C	B	+1	+2	+1									
B5	Balb/C	B	-/+	+2	+1	+1								
B6	Balb/C	B					+1/+1	+1/+2+3	+1/+1	+-/+2	+1/+2+3	+1/+2	+1/+2	+1/+2
B7	Balb/C	E	+1	+2	+2									
B8	Balb/C	E	-+	+2	+1	+1								
B9	Balb/C	E					-+/-+	+1+2/+2+3	+1/+1	+1/+2	+1/+2+3			+1/+2+3
B10	Balb/C	G	+1	+2	+2									
B11	Balb/C	G	-+	+2	+1									
B12	Balb/C	G					+1/+2	nd/+2	+1/nd	-+/+2	+1/nd			+1/+2+3
B19	Balb/C	mIgG	+1	+2	+1									
B20	Balb/C	mIgG	+1	+2	+1	+1								
B21	Balb/C	mIgG					+1/+2	+1/+1+2	+1/+2	+1/+1+2	+1/+1	+1/+1	+1/+1	+1/+1
B23	NZB/W	G^{biotin}	+3+4			+4								

The values were determined by dipsticks from Bayer Diagnostics.

4.4 Analysing serum samples from mice injected with antibodies

ELISA analysing serum level of injected anti-dsDNA antibodies

The results of the ELISA test to detect anti-dsDNA antibodies in sera showed some general tendencies. At the time prior to bolus injections, there were no anti-dsDNA antibodies to be detected in sera in the BALB/c mice. One hour after injection the level incidentally increased, and 24 hours after injection the antibodies were almost cleared from the circulation (Table 4.5 and Figure 4.7 A).

Table 4.5 Anti-dsDNA antibodies in sera after bolus injections.

			Titers of serum antibodies			
Mouse	mAb	Injection	0 hour$^{\Omega}$	1 hour	6 hours	24 hours
B1	C	0.5 mg	0	60%*	250	
B2	C	1.0 mg$^{\#}$	0	6400	2700	650
B4	B	0.5	0	55%*	270	
B5	B	0.5	0	2000	1500	220
B7	E	0.5	0	5400	2500	
B8	E	0.5	0	1350	1250	270
B10	G	0.5	0	150	350	
B11	G	0.5	0	225	600	30%*
B19	mIgG	0.5	0	10%*	0	
B20	mIgG	0.5	0	10%*	10%*	0

Titer values were defined as the point where dilution/titration curve are crossing the line for 40% max binding of a reference antibody. This cut-off value is set at OD 1. $^{\Omega}$Sample taken before injection. $^{\#}$ Mouse B2 got an injection of 1mg antibody. *Means % binding related to the cut-off level.

These findings are in accordance with proteinuria levels at the same point of time. Monoclonal antibody G showed divergent low values compared to the other mAbs and irrelevant mouse IgG showed binding levels fare below cut off value. Repeatedly injected mice showed the same pattern (Figure 4.7 B exemplified for mouse B12). The values increased one hour after injections, and decreased back to levels below cut-off by the time for the next injection (every 3 day).

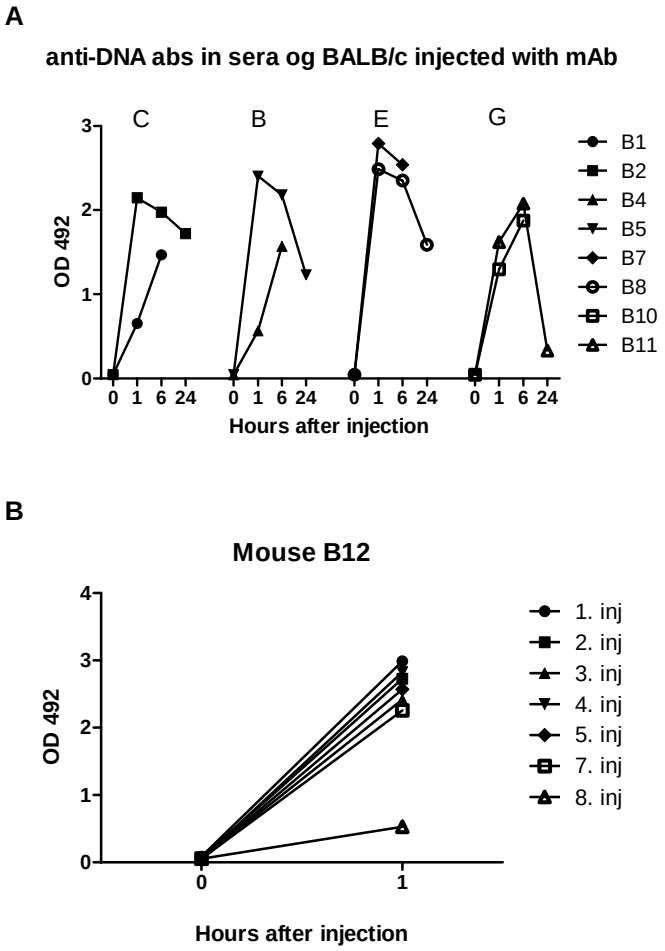

Figure 4.7 Analysis of serum anti-dsDNA antibody levels in BALB/c mice after injection of purified anti-dsDNA mAbs. A, Circulating anti-dsDNA antibodies measured after 0, 1h, 6 h and 24h after one bolus injection of indicated mAb. B, Serum anti-dsDNA antibodies of mouse B12 measured before and 1 h after each injection.

The reason why anti-dsDNA mAb was low 1 hour after the 8th injection is not known, but may be explained by accumulation of extra-cellular chromatin during treatment with antibodies.

Direct immunofluorecence microscopy

Direct immunofluorecence was performed on 4µm cryosections from kidneys of injected mice to detect in vivo bound autoantibodies and C1q, the first factor in the complement system. Each experiment included a panel of three mice representing the same injected antibody, one non-injected BALB/c mouse as negative control and one nephritic NZB/W mouse as positive control.

Deposits of IgG autoantibodies and C1q were evaluated and results are summarized in Table 4.6. The bolus mice showed none or little deposits of autoantibodies after 6 and 24 hours, and C1q was not present at this point of time. After 4 weeks though, repeatedly injected mice show autoantibody deposits and also co-localization of C1q together with the antibodies. This demonstrates that C1q are closely associated with IgG. This technique has the disadvantage of having low resolution. Still, for the bolus injected mice the granular deposits seem to be localized at capillary lumen and the repeatedly injected mice seem to have small additional deposits in mesangium. As an example, glomerulus of mice injected with mAb G is shown in Figure 4.8, and is representative for the other antibodies tested with this method. DIF was not an optimal technique because the resolution is low making it difficult to determining binding sites of antibodies, and the technique suffers from the problem of non-specific binding. We therefore went further to use high resolution technique.

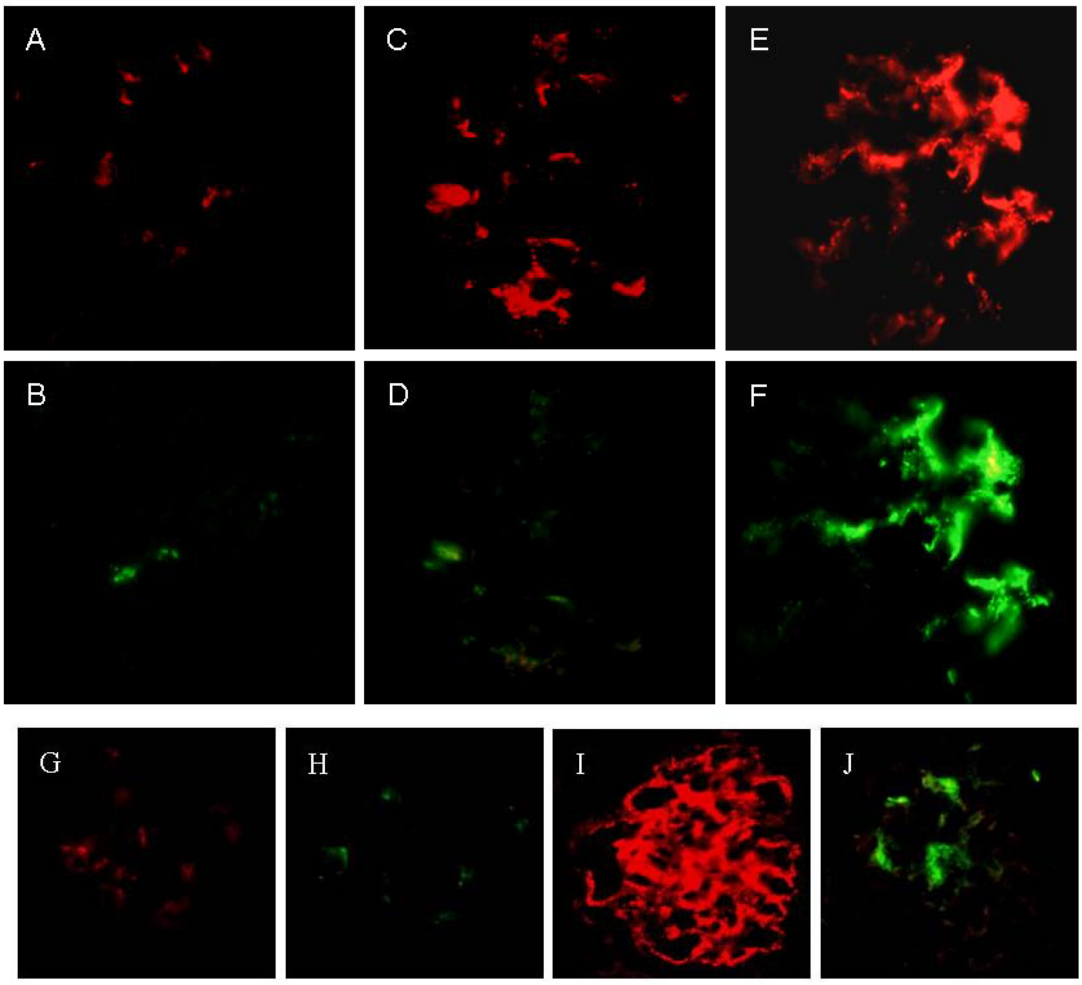

Figure 4.8 Direct immunofluorecence photographs of murine kidney sections.
Autoantibody deposits displayed in red colour (AlexaFluor 568-conjugated goat anti-mouse) and complement factor C1q in green colour (AlexaFluor 488-conjugated anti-rat). Mouse B10, bolus injected with mAb G and sacrificed after 6 hours (**A, B**). Mouse B11, bolus injected with mAb G and sacrificed after 24 hours (**C, D**). Mouse B12 repeatedly injected with mAb G (**E, F**). Non-injected BALB/c (**G, H**), nephritic NZB/W (**I, J**).

Table 4.6 Results of DIF and IEM of kidneys from injected BALB/c mice
Deposition of IgG and C1q detected by direct immuno fluorescent microscopy (DIF) and presence of immune complexes in electron dense structures (EDS) and capillary lumen by immune electron microscopy (IEM)

			Microscopy				
			Deposits				
			DIF		IEM		
Mouse	**Injected (mg)**	**mAb**	**IgG**	**C1q**	**EDS**	**Cap.Lum**	**other**
B1	0.5	C	nd	nd	÷	+	
B2	1.0	C	nd	nd	÷	+	
B3	0.2 x 8	C	nd	nd	+ +	+	collagen
B4	0.5	B	+	÷	nd	nd	
B5	0.5	B	+	÷	÷	+/ ÷	
B6	0.2 x 8	B	++	+	+	+ +	
B7	0.5	E	+	÷	÷	+/ ÷	
B8	0.5	E	+	÷	÷	+	
B9	0.2 x 8	E	++	+	+	+ +	granulocyttes
B10	0.5	G	÷	÷	÷	+/ ÷	
B11	0.5	G	+	÷	÷	+	
B12	0.2 x 8	G	++	++	+ +	+	granulocyttes/ collagen
B19	0.5	mIgG	nd	nd	÷	+	
B20	0.5	mIgG	nd	nd	÷	+/ ÷	
B21	0.2 x 8	mIgG	nd	nd	÷	÷	
Balb/c negative			+/÷	+ /÷	÷	÷	
B23 NZB/W	0.5	G^{biotin}	nd	nd	+ +		granulocyttes

The findings are visually evaluated by inspection of the micrographs, where (+/÷) represent insignificant or indistinct deposits, (+) represent moderate findings and (++) represent explicit findings. nd: not done.

Electron microscopy

The mice injected with mAbs were investigated by TEM. The morphology of endothelial cells and podocyte foot processes seem normal, so do the mass of the GBM and mesangium. The morphological changes observed were restricted to minor deposits of EDS along GBM in mice repeatedly injected with purified mAbs (Figure 4.9).

In vivo localization of autoantibody deposited in glomeruli was determined using co-localization electron microscopy. Ultrathin cryosections of 80nm was first immunostained to detect in vivo bound autoantibodies, visualized with PAG-5 (5nm gold). This was followed by co-localization of DNA, visualised by PAG-10 (10nm gold). Inspections of kidney sections of the bolus mice revealed no deposits or direct binding to membranes. However, there was minor amount of IgG molecules identified in the capillary lumen associated with a non-identified, but often seen, fluffy material (Figure 4.10). Traces of co-localized DNA were also observed in this material, indicating small amounts of circulating DNA-containing immune complexes. Mice injected repeatedly over 4 weeks also had fluffy material in capillary lumen. In contrast to the bolus mice, this was associated with explicit amount of autoantibodies, and co-localization IEM showed different levels of DNA, from high (Figure 4.12e) to minor, if any (Figure 4.13b).

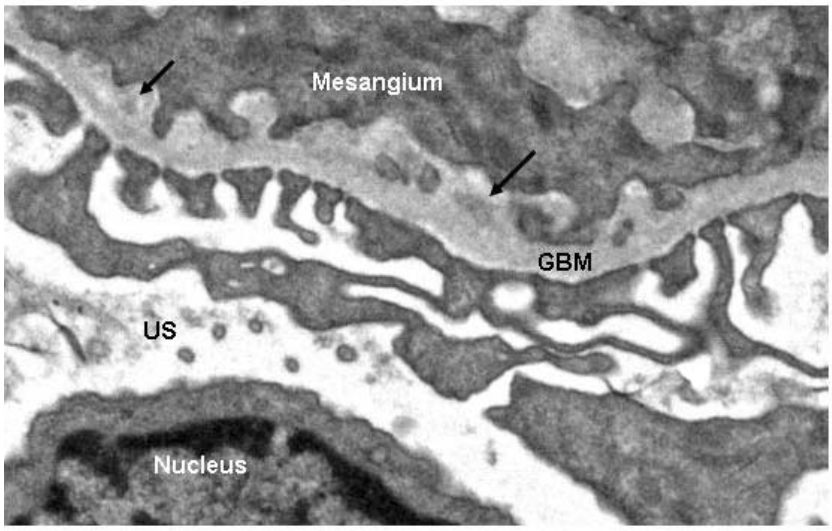

Figure 4.9: Mouse B12 injected with mAb G, 0.2 mg x 8. Glomeruli analysed by TEM. Minor deposits of EDS are shown by arrows. GBM; glomerular basement membrane, US; urinary space,

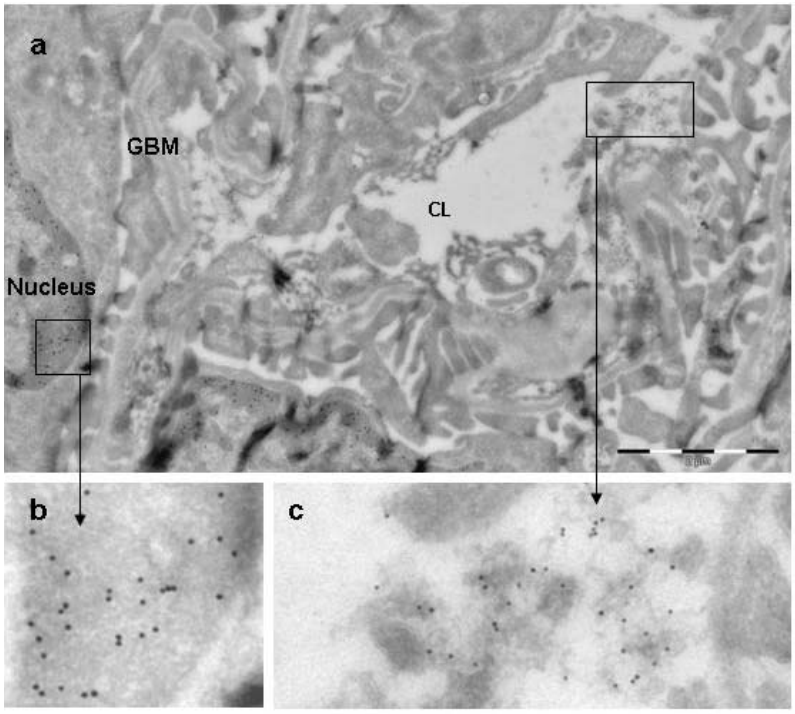

Figure 4.10: Mouse B1 injected with 0.5 mg mAb C. Glomeruli analysed by IEM/CIEM. Autoantibodies (shown as 5nm gold) were observed in capillary lumen associated with fluffy material (**a**, enlarged in (**c**)). Further analyzes showed no co-localization of anti-dsDNA 163p77 in this material; DNA was restricted to nucleus (shown as 10nm gold) (**b**). GBM; glomerular basement membrane, CL; capillary lumen.

Mice exposed to repeated injections had deposits in mesangium (Figure 4.11, 4.12 and 4.13) and additional subendothelial deposits associated with GBM (Figure 4.15). Although the deposits were relative small, they were explicitly accumulated as EDSs. All observations of deposited anti-dsDNA antibodies were in association with DNA visualized by PAG-10, except for mouse B6 (Figure 4.12 b). No loci were found only to bind mAbs and nucleus were the only site where DNA was detected alone by anti-dsDNA 164p77. Other observations of the glomeruli includes influx of granulocytes with vesicles containing autoantibodies (Figure 4.14 c and d) or including DNA (Figure 4.14 f), and 'microfilament-like structures' (collagen?) in mesangium associated with immune complexes visualized by PAG-5 and PAG-10 (Figure 4.11e). The negative controls, represented by healthy BALB/c mice and the mouse repeatedly injected with irrelevant mouse immunoglobulin (mIgG) showed no deposits (Figure 4.16). However, the bolus mice of mIgG had IgG in capillary lumen (Figure 4.16 a, c) and minor co-localization of DNA and IgG (Figure 4.16 a). The results of IEM are summed in Table 4.6, and examples are given in the following figures.

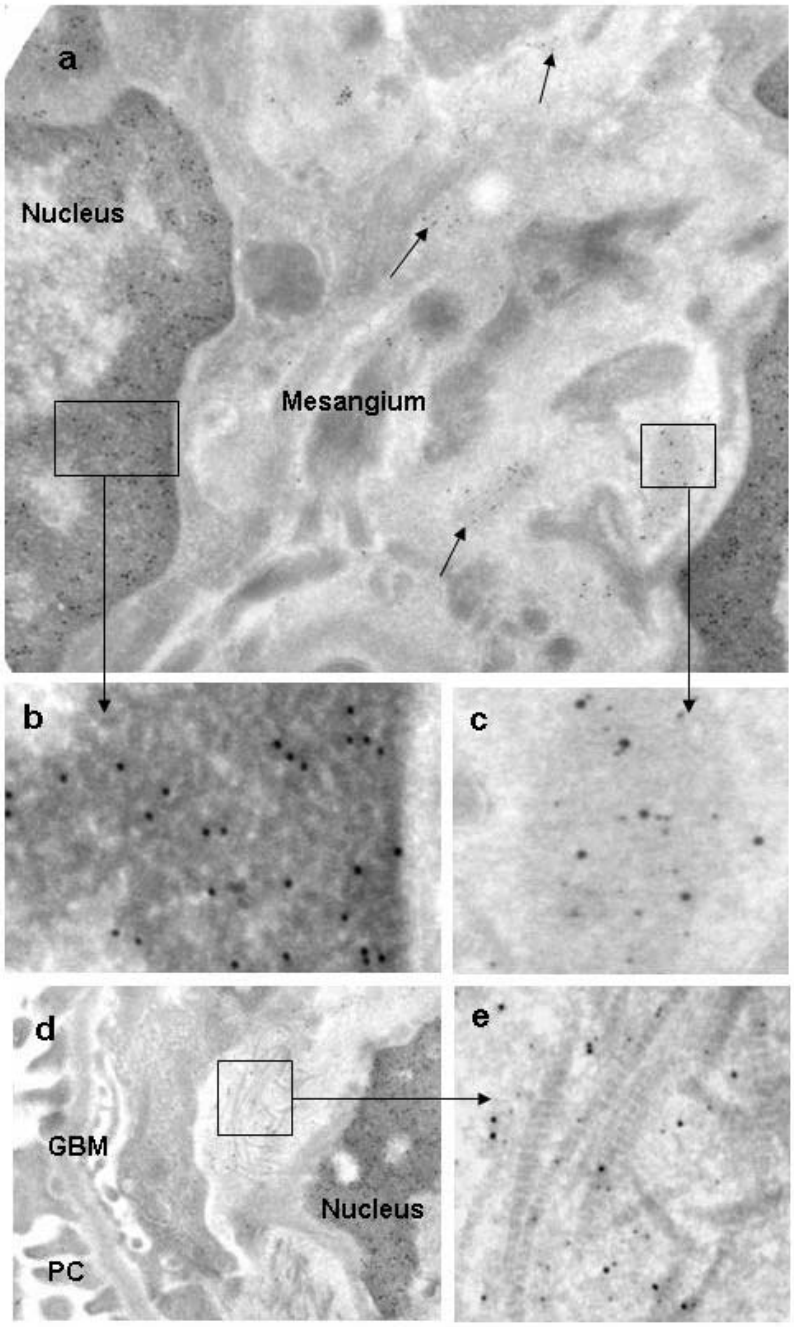

Figure 4.11: Mouse B3 injected with mAb C, 0,2 mg x 8. Glomeruli analysed by IEM/CIEM. Mesangium with electron dense structures (EDS), shown by arrows (**a**). Co-localization IEM analyses demonstrate autoantibodies traced by 5nm gold and anti-dsDNA 163p77 traced with 10nm gold in EDS (**a** enlarged in **c**). Anti-dsDNA 163p77 alone is restricted to nucleus (**a** enlarged in **b**). Deposits associated with collagen-like structures shown in (**d**) and enlarged in (**e**). No binding of autoantibodies was observed associated to the glomerular base membrane (GBM). GBM; glomerular basement membrane, PC; podocyte.

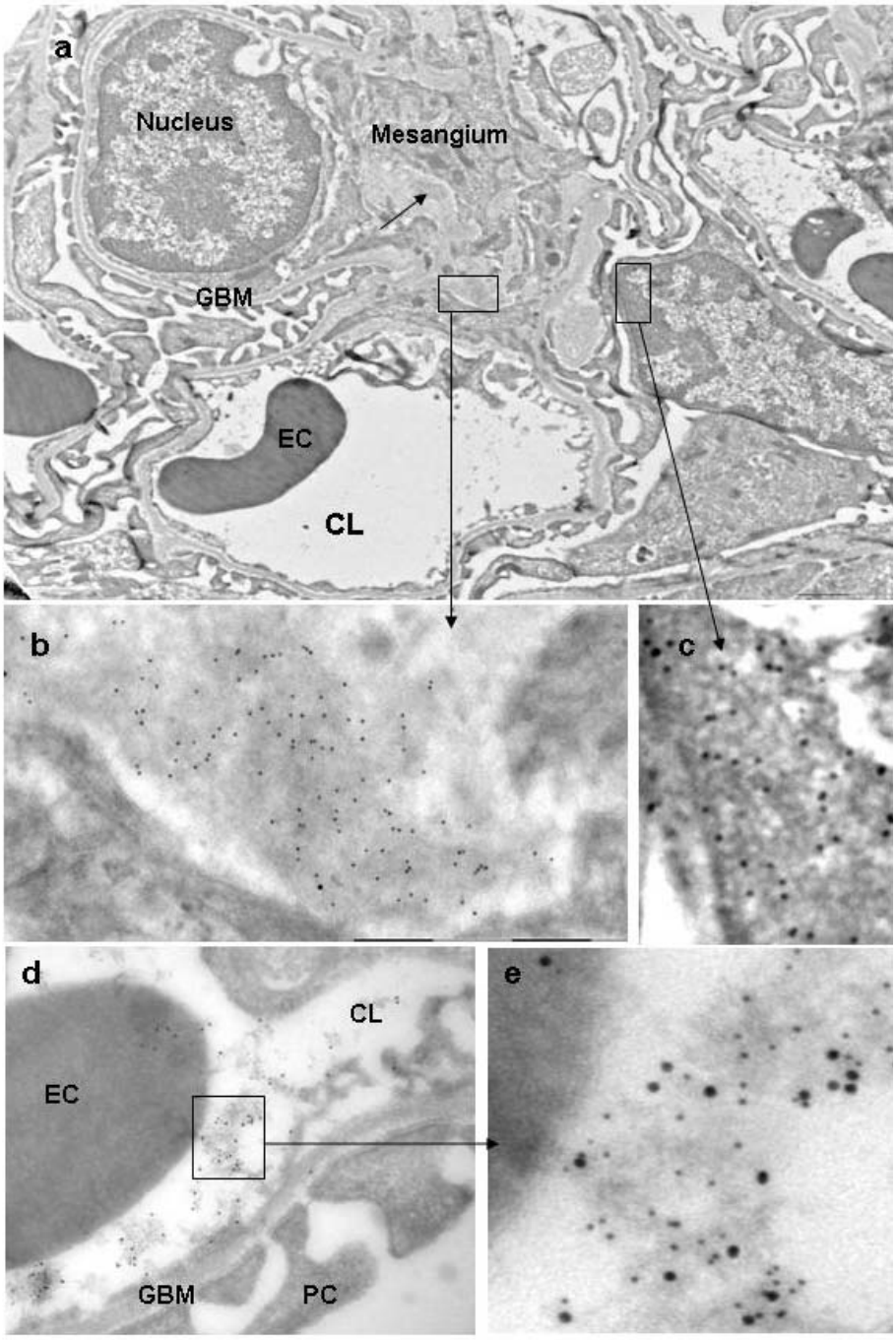

Figure 4.12: Mouse B6 injected with mAb B, 0,2 mg x 8. Glomeruli analysed by IEM/CIEM. Mesangium (**a**) with electron dense structures enlarged in (**b**). IEM analyses demonstrate autoantibodies (5nm gold) in these structures. Co-localization IEM identified DNA recognized by anti-dsDNA 163p77 (10nm gold) only in nucleus (**a** enlarged in **c**). Unidentified fluffy material in capillary lumen is associated with IgG (5nm gold) and DNA (10nm gold) shown in (**d**) enlarged in (**e**). GBM; glomerular basement membrane, CL; capillary lumen, EC; erythrocyte, PC; podocyte.

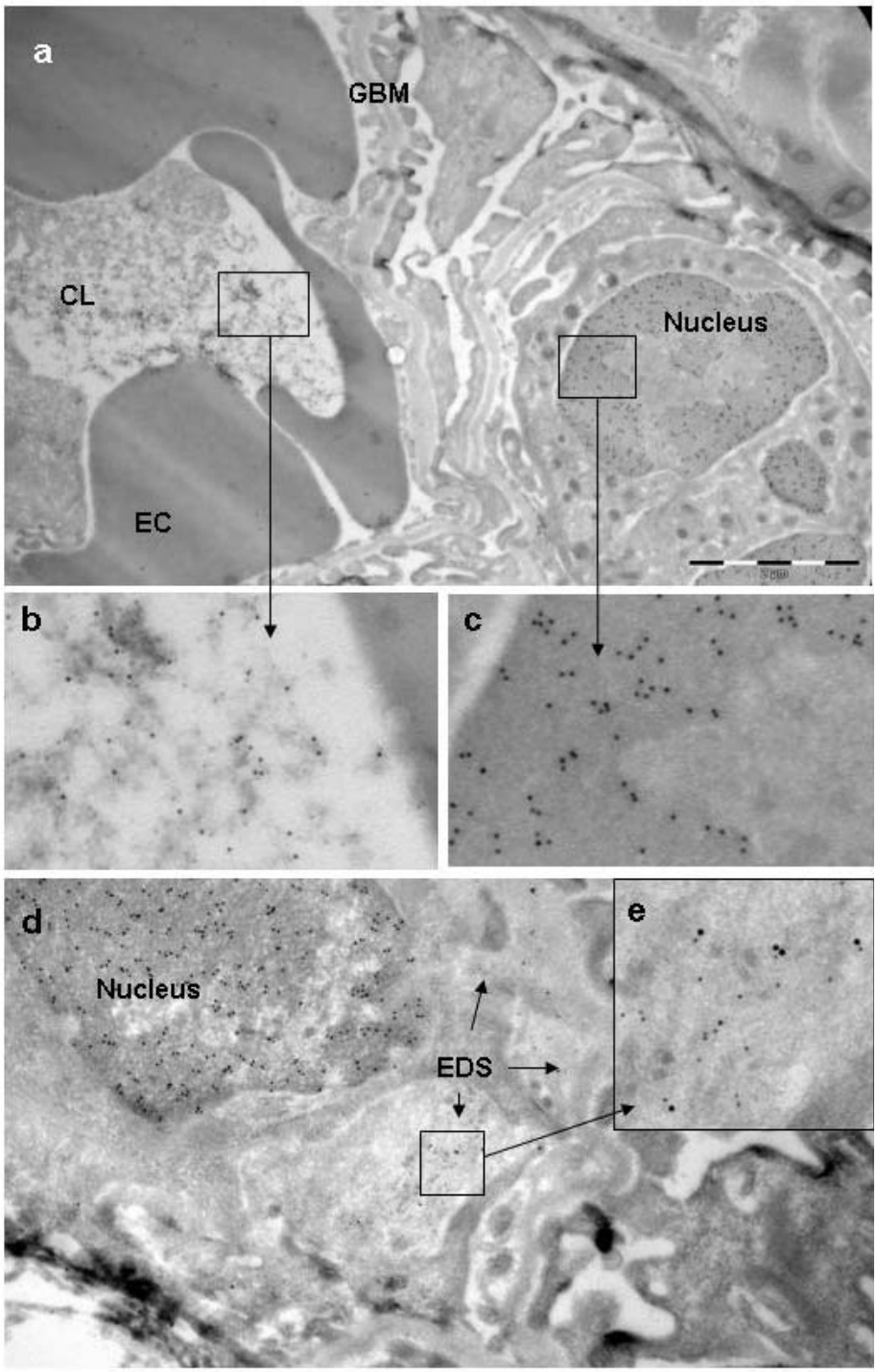

Figure 4.13: Mouse B9 injected with mAb E, 0.2 mg x 8. Glomeruli analysed by IEM/CIEM. IEM analyses demonstrate autoantibodies (5nm gold) in fluffy material in lumen, (**a**) enlarged in (**b**). Co-localization IEM identified DNA recognized by anti-dsDNA 163p77 (10nm gold) in nucleus (**a** enlarged in **c**) and minor deposits in capillary lumen (**a** enlarged in **b**). Electron dense structures, (**d**) enlarged in (**e**), was shown by IEM analyses to contain in vivo-bound IgG (5nm gold) and co-localization of DNA recognized by anti-dsDNA 163p77 (10nm gold). GBM; glomerular basement membrane, CL; capillary lumen, EC; erythrocyte, EDS; electron dense structures.

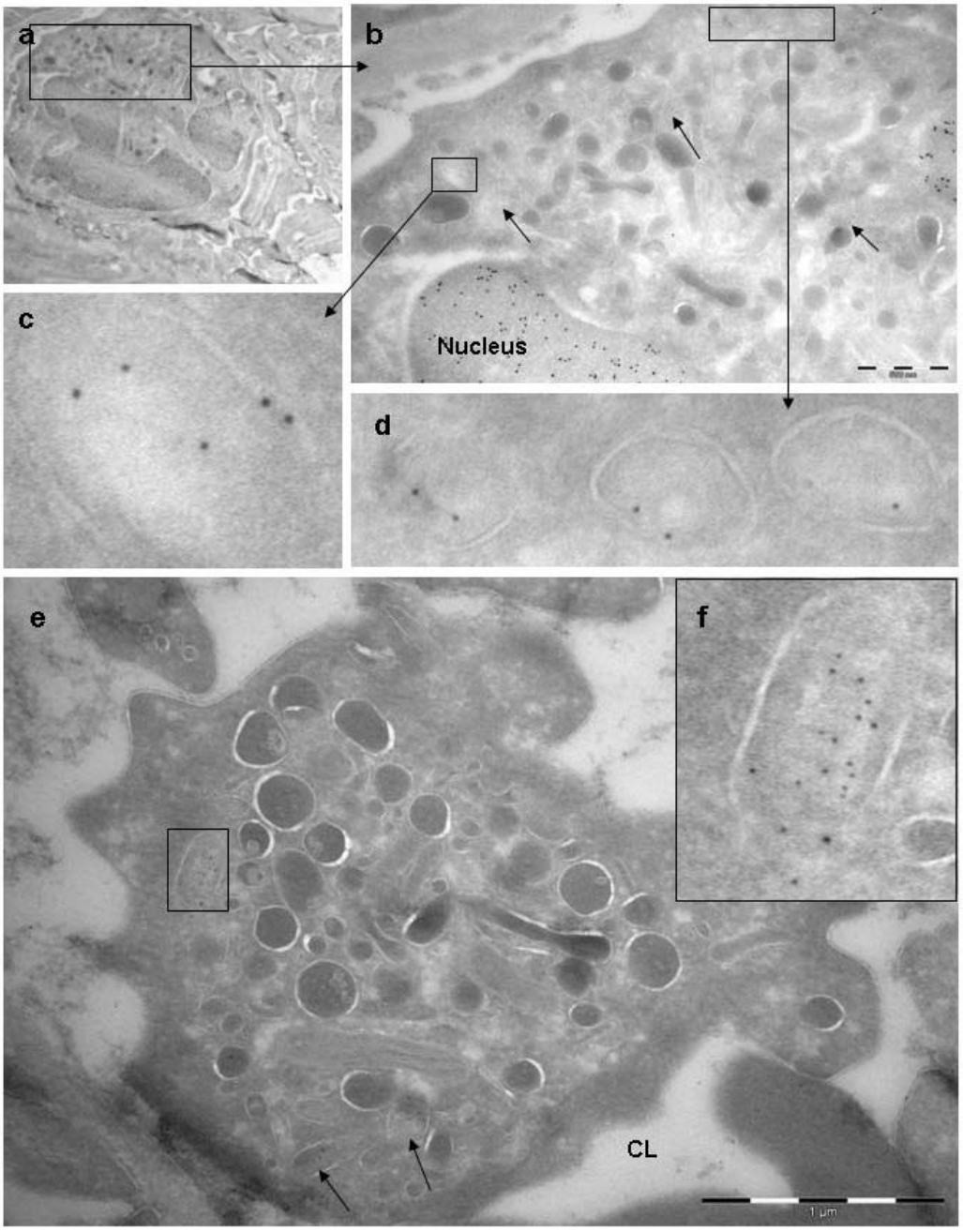

Figure 4.14: Mouse B9 injected with mAb E, 0.2 mg x 8. Glomeruli analysed by IEM/CIEM. Phagocytic granulocyte within glomerulus characterised with lobular nucleus and cytoplasmic granules filled with degradative enzymes, (**a**) and (**e**). IEM revealed in vivo-bound IgG (5nm gold) in vesicles of granulocytes (**b**) and enlarged in (**c**) and (**d**). Anti-dsDNA 163p77 (10nm gold) was shown in nucleus (**b**) and traces in vesicles (**f**). CL; capillary lumen.

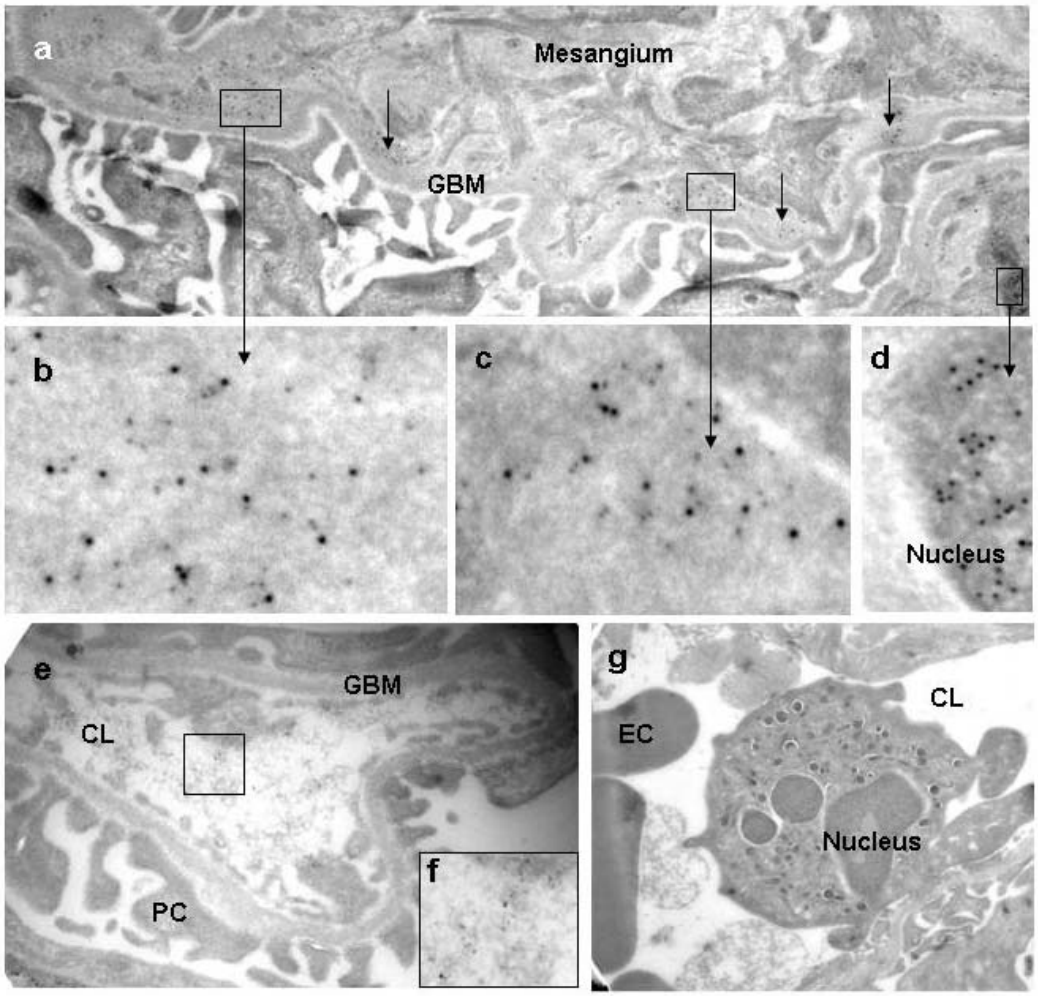

Figure 4.15: Mouse B12 injected with mAb G, 0,2 mg x 8. Glomeruli analysed by IEM/CIEM Mesangium with EDS associated with GBM as shown by arrows (**a**). By IEM autoantibodies was traced by 5nm gold and co-localization IEM showed binding of anti-dsDNA163p77 traced by 10nm gold in EDS (**b**) and (**c**). Anti-dsDNA 163p77 (10nm gold) alone was only shown to bind in nucleus (**d**). No binding of IgG to the GBM was observed. Fluffy material in capillary lumen was associated with autoantibodies (5nm gold) and DNA (10nm gold) shown in (**e**) enlarged in (**f**). Granulocytes were observed in glomerulus. GBM; glomerular basement membrane, PC; podocyte, CL; capillary lumen, EC; erythrocyte.

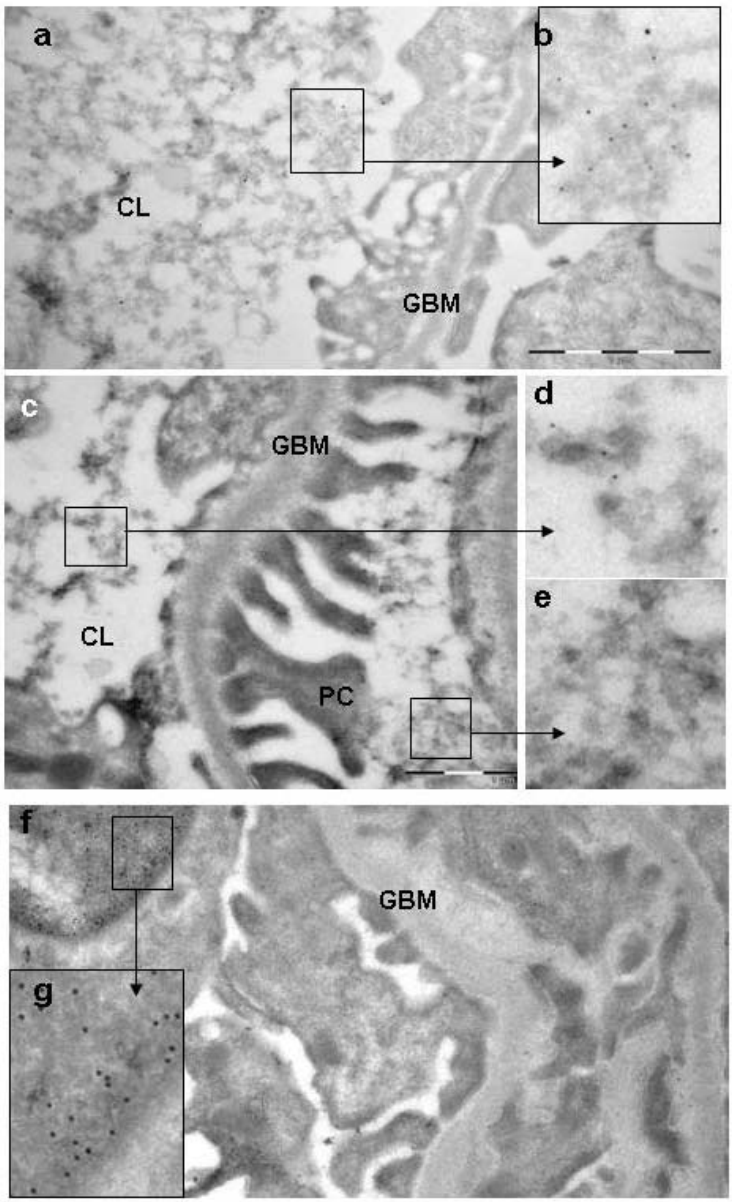

Figure 4.16: Mice injected with polyclonal mouse IgG and analysed by IEM/CIEM. In mouse B19 (injected 0.5 mg) IEM analyses identified few IgG (5nm gold) in fluffy material in capillary lumen (**a**) enlarged in (**b**). In B20 (injected 0,5 mg), IEM identified minor amount of IgG (5nm gold) associated with fluffy material in capillary lumen (**c**) enlarged in (**d**). In the same mouse, fluffy material was observed in the urinary space and this was not associated with autoantibodies or DNA (**e**). In B21 (injected 0,2 mg x 8), no observation of IgG was done. Co-localization IEM identified DNA (traced with anti-dsDNA163p77 and 10nm gold) in nucleus alone, (**f**) enlarged in (**g**). GBM; glomerular basement membrane, PC; podocyte, CL; capillary lumen.

Additionally, a sample of purified antibody mAb G was biotinylated prior to injection into NZB/W mouse to target the exact deposit compared to the inherently bound autoantibodies. The lupus prone NZB/W mouse got injected the same amount as the other bolus mice and was sacrified after 24 hours. The inspection of the glomerulus revealed presence of EDS along GBM, containing inherent autoantibodies visualised by PAG-5 and injected autoantibodies detected by IgG anti-biotin antibodies and PAG-10 (Figure 4.17 C), but also EDS exclusively containing injected autoantibodies (Figure 4.17 B). It seems as the latter are in closer association to subendothelial location in GBM (Figure 4.17 D enlarged in F). Macrophages with vesicles containing autoantibodies (5nm gold) and injected biotinylated IgG anti-dsDNA mAbs (10nm gold) were also seen (Figure 4.17 D enlarged in E)

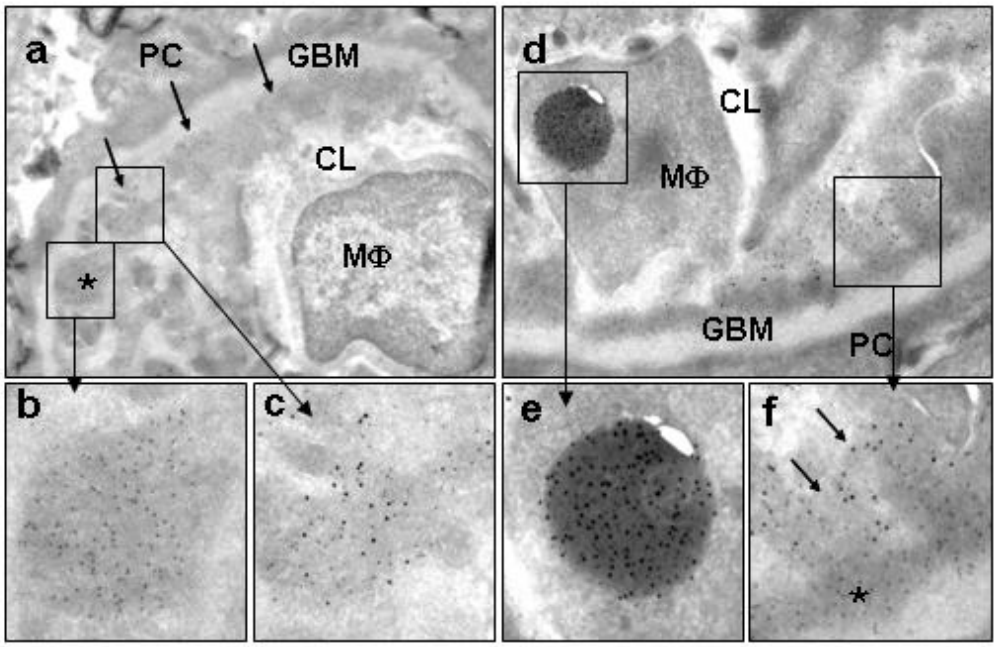

Figure 4.17: Glomeruli of NZB/W mouse B23 injected with biotinylated mAb G analysed by IEM/CIEM. EDS localized along GBM and in mesangium matrix are demonstrated by arrows (**a**). EDS deposits was shown to contain inherent autoantibodies (5nm gold) (**b**), or deposits containing inherent autoantibodies and injected mAb (10nm gold) (**c**). Vesicles with co-localized inherent and injected autoantibodies are shown in (**d**) (enlarged in (**e**)). In (f) a gradient of deposits are shown; injected mAb are deposited closest to the capillary lumen and inherent autoantibodies in more interior areas of mesangium. (*exclusively 5nm gold). PC; podocyte, CL; capillary lumen, GBM; glomerular basement membrane, MΦ; macrophage

4.5 Data of the coded monoclonal anti-dsDNA antibodies

After closing the experiments, the blinded data of the mAbs was revealed. The general structural characteristics of the anti-dsDNA mAb included in this study are presented in Table 4.7. The database show that we have been working with four different hybridomas, three originate from the same mouse (no.163). DNA6 (= mAb G) show abnormal size of the light chain and diverging binding properties, but this have no obvious connection to any structural characteristics listed in Table 4.7

Table 4.7 Structural characteristics of the variable region of monoclonal anti-dsDNA antibodies.

mAb	Hybridoma	Isotype	V_H	D_H	J_H	V_L	J_L
B	163.87	IgG2a	V_HQ52	KGL**RR**AG	4	V_λ-2	2
C	163.119	IgG2a	V_HS102	DPYG**R**T**R**T	4	V_λ-10	1
E	163.132	IgG2b	V_H7183	HYYGS**R**TY	2	Vκ-8	2
G	DNA6	IgG2a	V_H558	E**R**TGTG	3	Vκ-8	1

Isotype is decided by the type of heavy chain. The V_H gene family for the V_H gene used to encode each mAb is indicated in the column V_H, and include CDR 1 and CDR 2. D_H is including the CDR 3 region, and shows the content of arginines (R) in the amino acid sequence. The light chain V segment is coded by V kappa and lambda genes and J genes.

5 Discussion

Anti-dsDNA antibodies are more frequent in SLE patients than lupus nephritis is (Balow et al., 2004). This indicates that not all anti-dsDNA antibodies are nephritogenic. There must be structural or molecular properties among sub-populations of anti-dsDNA antibodies that determine their pathogenic potensiale. Features as high affinity and specificity for DNA (Rekvig and Nossent, 2003) or cross-reactivity with inherent renal molecules (D'Andrea et al., 1996; Raz et al., 1993; Mostoslavsky et al., 2001; Raz et al., 1989; Raz et al., 1993) have been associated with glomerulonephritis, so has the availability for target antigens like chromatin fragments (Berden and van Bruggen, 1997; Kalaaji et al., 2007).

Sub-populations of anti-dsDNA antibodies have the potential to induce nephritis (van Bruggen et al., 1997b; Madaio and Shlomchik, 1996; Ohnishi et al., 1994; Raz et al., 1993; D'Andrea et al., 1996; Waldman and Madaio, 2005). However, there is no known marker to identify pathogenic/non-pathogenic antibodies involved in nephritis. There have been several different approaches to investigate the mAb glomerular binding properties in vivo, including perfusion studies and intraperitoneal injections of hybridomas (Dang and Harbeck, 1984; Ehrenstein et al., 1995; van Bruggen et al., 1997a; Kramers et al., 1994; Ohnishi et al., 1994; Tsao et al., 1992; Mostoslavsky et al., 2001). The approach presented in this study has the focus on injection of pure mAb followed by analyses of kidneys at the ultrastructural level.

The purpose for this study was to analyse the characteristics of antibodies that bind, respectively do not bind in vivo in the glomeruli, and the nature of their glomerular target structures. This study attempts to provide direct evidence for the pathogenic role of anti-dsDNA antibodies.

Properties of purified anti-dsDNA antibodies

Initially a panel of four coded mAb with distinct anti-dsDNA specificities was purified. Decisive for the outcome was the use of high salt concentrations to remove nucleosomes bound to mAbs in cultures of hybridoma cells and DNase to further remove chromatin. This method was shown to be effective. No contamination accounting for proteins or DNA could be seen in the purified IgG populations. The importance of removing chromatin is critical to avoid formation of immune complexes which may interfere with the binding of antibodies in kidneys (Kramers et al., 1994).

The purified mAb was tested in vitro for specificity, and we found that all mAb recognized DNA, nucleosomes, matrigel and phosphatidylserin, with the highest titers for DNA and lowest for matrigel. The analyses revealed individual differences among the mAbs, and antibody G had diverging results in the sense of particular lower binding capacity for all antigens except DNA.

Anti-dsDNA antibodies have specificity for dsDNA, but not necessary high affinity for that antigen. By surface plasmon resonance all antibodies showed significant affinity for DNA. MAb G and mAb C had especially high affinity, with k_D of 10^{-11}, and mAb E the lowest with k_D of 10^{-9}. The difference in affinity is 100-fold, but the variations among the antibodies may not be the important issue, but the fact that all bound DNA firmly. Thus, in principle, we could not pin-point any binding pattern that obviously separated the individual antibodies from each other with respect to nephritogenicity.

Binding of purified anti-dsDNA antibodies in vivo

At an ultrastructural level, distinct location of glomerular target structures for in vivo bound mAb was investigated. By TEM, minor morphological changes were observed as EDS in glomerulus. IEM was performed, and we made significant observations: After 4 weeks, all four injected sub-populations of mAb antibodies had deposited in glomeruli, and the deposits were localized as EDS in mesangial matrix and associated to the mesangial site of GBM. In bolus injected mice, deposits or other pathological changes could not be seen, neither after 6 hours nor after 24 hours, when the injected antibodies were not anymore detected in sera. This indicates that the attempt to reveal structural differences in mAb that correlate with differences in antigen binding in vivo could not be identified. All mAbs bound, including mAb G, and there is no property from Table 4.7 that stands out as responsible for this: all mAb had different germline genes and number of arginines in CDR3 and combinations of these.

It has been postulated that high affinity for dsDNA is a marker for nephritic antibodies but this has never been proven true (Isenberg, 1997; Wellmann et al., 2005; Izui et al., 1976; Suzuki et al., 1993). Our results showed highly different affinities for all mAbs and all were binding in vivo. These facts points at affinity as a poor marker for pathogenicity. This is in line with experiments performed in our group, which shows that anti-dsDNA antibodies in renal eluates from different nephritic mice with severe proteinuria vary from low to very high

affinity, indicating that antibody affinity is not a nephritogenic parameter (Mjelle et al., manuscript submitted)

Accumulation of arginines in the CDR3 of Ig heavy chain region is predicted to increase avidity (Ohnishi et al., 1994; Krishnan et al., 1996; Tillman et al., 1992)) According to this theory, we would expect higher affinity for mAb B and mAb C because of the two arginines, compared to mAb E and mAb G with one arginine residue, but this does not agree with our results. However, other charged amino acids or internal order in the chain may be of importance (Peeva and Diamond, 2004; Radic and Weigert, 1994)

In this study pathogenicity of anti-dsDNA antibodies is defined as the ability to induce nephritis in healthy BALB/c mice. An unknown number of the antibodies selected for us by Dr. Marion are supposed to be pathogenic. From the Table 4.3 and Table 4.7 we can not predict if any of these are pathogenic, but from the fact that the four mAbs we included in the study bound in glomerulus, we may predict that these have pathogenic potential. This would point at availability of chromatin fragments in glomeruli as a decisive pathogenic factor (Mortensen et al., 2008).

DIF, an inaccurate approach to study glomerular deposits compared to EM

By direct immuno fluorescent microscopy, deposits of IgG in repeatedly injected mice were confirmed, but there was staining in glomerulus in bolus injected mice as well. However, inspection of IEM micrographs revealed true deposits only in repeatedly injected mice. What seem like GBM deposits in bolus injected mice by DIF may in fact be IgG associated with fluffy material within capillary lumen. This may well be complexes of injected IgG and circulating small chromatin fragments. This leaves DIF a rough method for inspection of in vivo deposits, and precautions must be taken before drawing any conclusions based solely on this method. Different studies have based their conclusions upon results by DIF, see e.g. (Ohnishi et al., 1994; van Bruggen et al., 1997a; Kramers et al., 1994).

All mice with IgG deposits showed strict co-localization with C1q by fluorescent microscopy after 4 weeks. This may indicate onset of inflammatory responses promoted by complement activation. By IEM, granulocytes were shown to be present in glomeruli. C1q interactions are mainly mediated through the constant region (Fc) of antibodies and complement receptors on different types of effector cells. Phagocytic cells ingest immune deposits, as demonstrated in this study by granulocytes containing closed vesicles with anti-

dsDNA antibodies or immune complexes (Figure 4.14 d and f)). The distribution of the deposits within glomerulus reflects the course of the disease. We traced the deposits to mesangium and GBM associated subendothelial areas. This location allows recruitment of effector cells from the vascular space and initiation of inflammatory responses (Wener and Mannik, 2004).

Important to notice from inspection of glomeruli by DIF of serial injected mice, are the lack of even distribution of deposits throughout the glomerulus, but restricted to scattered areas. This may reflect the early phase of the disease with the glomerulus not 'filled up' with deposits. This observation may be of great importance for evaluating IEM results; the selected areas of sections may have great impact of the result. A kidney from BALB/c mouse has 200 glomeruli, and usually there is only 0.5-2 glomerulus pr section. To get representative information, the slices should be taken from different parts of the kidneys, which we did not. It is interesting to note, that distribution of proliferative lesions in WHO class III and IV lupus nephritis, are highly irregular both within and amongst glomeruli (Balow et al., 2004). This could reflect the pattern of deposits from which the lesions grow.

Is deposition of IgG–chromatin complexes sufficient to promote lupus nephritis?

In contrast to the theory of nephritogenic anti-dsDNA antibodies, the occurrence of EDS without proteinuria point at other factors for inflammatory onset. Clinical nephritis is defined as 2+ or higher proteinuria on at least two consecutive measurements (Ohnishi et al., 1994) or 3+ according to ACR criteria. Only one of the serial injected mice showed signs of high proteinuria and only at the end point. We did not find any correlation between proteinuria and presence of EDS in the injected mice. For all the mice, we observed moderately increased protein levels in the urine samples one hour after injection and subsequently decreasing by the time for the next injection. The bolus injected mice injected with even higher amount of antibodies (0.5mg), also showed transient proteinuria. This suggests that the filter unit is incidentally clogged, but able to restore between injections, and the mice were sacrificed before onset of eventual sustainable proteinuria. An additive explanation may be the early phase of disease; the deposits are small and damage to glomerulus is not reflected as proteinuria yet. It would be interesting to see further development of the symptoms, with or without more injections. The potential nephritic processes are in an early stage and not easily

detected. Time for sacrificing must be discussed for further studies, and also the number of injections may prove important.

The same pattern as in proteinuria was shown for mAbs in serum; increasing levels one hour after injection, followed by disappearance from circulation after 6h (24h-2days). So, after one hour, we observed transient levels of antibodies in sera and also transient proteinuria. Where do the antibodies go? One explanation may be clearance of large immune complex by the Kupffer cells in the liver and phagocytes in the spleen. Only very small immune complexes may remain in circulation and possible accumulate in kidneys through e.g. binding to Fc-receptors on mesangial cells (Wener and Mannik, 2004).

The time aspect from appearance of in vitro-bound immune complexes to clinical nephritis might be explained by gradual accumulation of antibodies. After initial deposition in glomerulus, provided sustained injections of the autoantibodies, the small immune complexes undergo further condensation into even larger structures. Eventually, the deposited autoantibodies reach a level where they are able to activate the complement system. In mice that got bolus injections, the immune complexes might have been below a detection level. After repeated injections, (and totally three times more mAb injected), accumulated anti-dsDNA antibodies are able to start the inflammatory machinery (experiments in progress).

Anti-dsDNA antibodies recognize DNA containing structures in glomeruli

The ability to cross react with inherent glomerular antigens is a known property of anti-dsDNA antibodies, but this is not a feature proven to be connected to pathogenicity. Experiments arguing for (Mostoslavsky et al., 2001; D'Andrea et al., 1996; Raz et al., 1993) and against (Ohnishi et al., 1994; Berden et al., 1999; Kalaaji et al., 2006b; Kalaaji et al., 2006a) reflects this dilemma.

By ELISA we collectively tested laminin, collagenIV and heparan sulphate proteoglycan, using matrigel as antigen. These molecules are rich in repeated, negatively charged units suitable for binding the cationic residues of anti-dsDNA antibodies, (Sabbaga et al., 1989; Amital et al., 2005; Suzuki et al., 1993). All tested anti-dsDNA antibodies bound matrigel *in vitro*, and this polyreactivity may support the hypothesis of cross reactive mediated binding to glomerular membrane structures.

On the other hand, *in vivo* studies did not support this result. IEM using experimental antibodies allowed us to detect the in vivo bound antibodies and the co-localized distribution

of DNA. The results revealed binding of anti-dsDNA antibodies together with DNA, and this was restricted to EDS. The exception was B6, which EDSs was shown to contain only autoantibodies. Maybe DNA in these EDS is saturated by in vivo-bound antibodies. A TUNEL assay may confirm whether EDS in B6 consist of chromatin. EDS are believed to consist of immune complexes composed of anti-dsDNA antibodies and nucleosomes (van Bruggen et al., 1997a). Since the injected mAbs bind in vitro to DNA and matrigel, it would be interesting to test for presence of pure glomerular antigens in EDS. Kalaaji have tested for laminin and collagen and found it not to be associated with EDS (Kalaaji et al., 2006a).

Nucleosomes are shown to have high affinity for GBM (Mjelle et al., 2007), and electrostatic forces are suggested to be responsible for the nucleosome-mediated binding of nucleosomes in complex with IgG to GBM. The positively charged histone tails of the nucleosome attracts to negative charged GBM components. Binding of anti-dsDNA antibodies will decrease the density of negative charges of the nucleosome. This will enhance binding of the complex to the negatively charged GBM structures and lead to nucleosome-mediated binding (Kramers et al., 1994; van Bruggen et al., 1997b; Raats et al., 2000).

Immune complex are formed by anti-dsDNA antibodies bound to nucleosomes. It is not proven where these immune complexes are formed. Some results suggest that this happened locally in diseased kidneys (Makino et al., 2003; Mannik et al., 2003). Other claim that these complexes come from circulation based on finding of DNA- anti-DNA complexes in sera of lupus patients (Kramers et al., 1994; van Bruggen et al., 1997b; Pisetsky, 2004b). This fits with observations in our study, - the location of EDS in the vascular area and the presence of fluffy material in capillary lumen staining positive for DNA and injected anti-dsDNA mAbs. Additionally, the location of deposits of injected biotinylated anti-dsDNA mAbs in mice seems to reveal a ‘two-wave’ intruding of antibodies into mesangium, with the injected antibodies in the area closest to GBM as latest arrived antibody from the circulation.

Availability of chromatin in glomeruli, - a key pathogenic factor

Accumulation of nucleosomes is assumed to be linked to disturbed apoptosis or reduced clearance of apoptotic material and this have been described in both murine and human SLE (Dieker et al., 2004; Berden, 2003; Makino et al., 2003; Perniok et al., 1998; Gaipl et al., 2005; Herrmann et al., 1998).

Nucleosomes are target structures for anti-dsDNA antibodies, and originate from apoptotic cells. Kalaaji performed a convincing experiment showing the nature of the chromatin complexed in EDS and its possible origin. By TUNEL assay for detecting apoptotic extracellular nucleosomes, biotinylated nucleotides were incorporated into ends of nicked DNA. This assay was applied to co-localization IEM allowing simultaneous detection of intra-cellular DNA and in-situ bound autoantibodies (Kalaaji et al., 2007).

Because all autoantigens targeted in SLE, including nucleosomes, are located in apoptotic blebs at the surface of apoptotic cells, it has been postulated that a decreased phagocytosis of these blebs may be a pivotal feature in an autoimmune response (Casciola-Rosen et al., 1994). The process of phagocytosis of apoptotic cell is initiated by a number of surface changes, and expression of phosphatidylserine is one of the most important. Via β2-glycoprotein I, which bind PS, the apoptotic cell binds to the macrophage and is internalized and degraded (Savill and Fadok, 2000). The anti-dsDNA antibodies are thought to have structural compatibility for phosphodiester groups common to both DNA and phospholipids (Figure 5.1) (Lafer et al., 1981). Anti-dsDNA antibodies are suspected to mask the structures for phagocytosis-related receptors and prevent recognition and phagocytosis (Mevorach, 2000; Manfredi et al., 1998; Cocca et al., 2001), and in this way contribute to increased levels of extra-cellular nucleosomes. This is in line with the fact that antibodies against different types of phospholipids are often associated with SLE (Hasselaar et al., 1990). We tested the anti-dsDNA antibodies' ability to bind PS and PC, and all showed moderate binding to these phospholipids. We may speculate if binding of these purified antibodies to apoptotic cells in vivo disturb the phagocytosis, leading to gradually increased release of nucleosomes followed by immuno complex formation and deposits as we observed in serially injected mice.

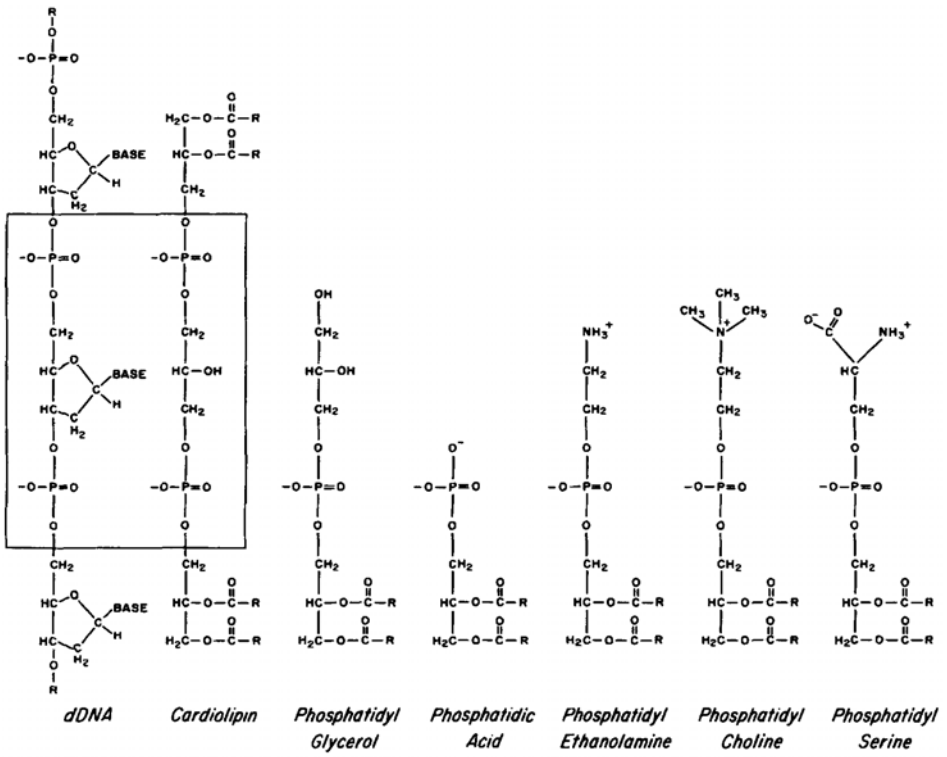

Figure 5.1. Comparison of chemical structures of DNA and phospholipids (Lafer et al., 1981)

C1q is an important factor in clearance of apoptotic material including chromatin by binding apoptotic cells and enhance their uptake by phagocytes (Mevorach, 2000) as well as bridging immune complex and scavenger receptors. Deficiency to C1q is associated with SLE (Walport et al., 1998). On the other hand, C1q is a strong inducer for inflammation. By DIF it was demonstrated that C1q are closely associated to IgG and not to apoptotic cells, pointing at the pathogenic role of anti-dsDNA antibodies as activator of the complement cascade, and secondary the macrophage and granulocyte invasion. Availability of chromatin in anti-dsDNA antibody positive individuals may therefore activate the inflammatory machinery, resulting in e.g. nephritis or dermatitis typical for SLE.

The role of nucleosomes as inducer and target structures for potentially nephritogenic anti-dsDNA antibodies

The hypotheses of the formation of anti-dsDNA antibodies and their role in the pathogenesis of lupus nephritis are present in Figure 5.2. Nucleosomes are the driving antigen

in SLE responsible for the induction of the autoimmune response, but also responsible for targeting the anti-dsDNA antibodies to the GBM.

We injected pure antibodies into healthy mice. This means that the deposited immune complexes must have been formed with targets present in the body. Availability of target antigens may be the critical parameter for deposits (van Bruggen et al., 1997b) and increased level of circulating nucleosomes has been seen in SLE patients (Rumore and Steinman, 1990; Decker, 2006; Koutouzov et al., 2004). Maybe the level of nucleosomes present in circulation of our experimental mice is too low to bind the relative large dose of anti-dsDNA antibodies injected, and this reflects the poor binding in the bolus injected mice. After repeated injections, an immunologic response may occur that generates more apoptotic cells, and nucleosomes are released and available for complex formation. The autoimmune NZB/Z mouse which was bolus injected with biotin-conjugated mAb showed deposits of the injected antibody after 24 hours. This mouse already produces autoantibodies and had heavy deposits, so the target (nucleosomes) must already be present in circulation and in situ.

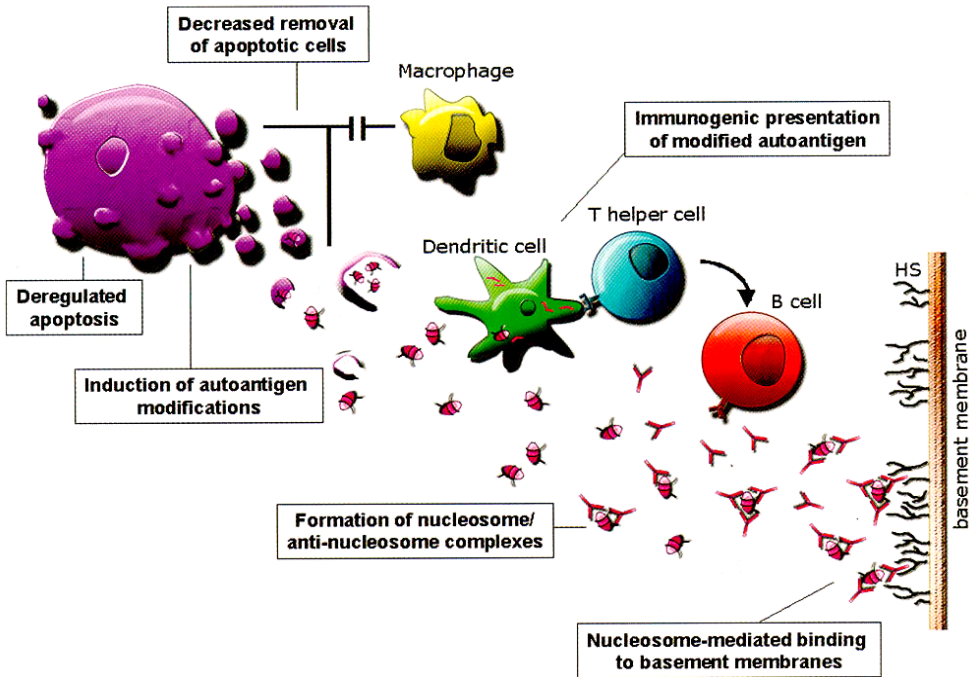

Figure 5.2: Nucleosomes as inducer and target for anti-dsDNA antibodies.
Reduced phagocytosis of apoptotic cells leads to release of nucleosomes. Antigen presenting cells (dendritic cells) present nucleosomes for preactivated autoreactive T cells which stimulates B cells to produce anti-dsDNA antibodies. Immune complex between these antibodies and nucleosomes in circulation are formed and this is binding to basement membrane in glomerulus. This activates complement which induces local inflammation; lupus nephritis (Figure taken from CC van Bavel, 2007 *In* Autoantibodies 2.ed, ISBN: 978-0-444-52763-9)

Concluding remarks

There is no known marker to identify pathogenic antibodies involved in nephritis. All tested anti-dsDNA mAbs had the potential to deposit in glomeruli, even in healthy mice. This lead to the assumption, that nephritic potential may not be connected to structural characteristics of particular anti-dsDNA antibody sub-populations. It seems like anti-dsDNA antibodies in general, and not specific sub-populations, are involved in development of lupus nephritis.

There is a growing evidence for nucleosomes to be the inducer and target for anti-dsDNA antibodies, and if availability of nucleosomes is the critical parameter for developing glomerulonephritis, there is no need to consider whether there exist particular pathogenic antibody sub-populations. Solving this issue may lead to new approaches in diagnostics and therapies of lupus nephritis.

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7 Appendix

7.1 Protocols

Purification of monoclonal antibodies

1. Transfer 10 ml hybridoma cell culture supernatant to each Protein-A-Sepharose column. Seal off both ends with parafilm, and incubate over night at 4°C at rotation. Centrifuge at 4000 rpm for 4 minutes. The antibodies are supposed to bind to the beads, and the supernatant is discarded. (Aliquot 1)

2. Wash the column with 10 ml High Salt Solution (3 M NaCl in PBS, pH 7.2). Centrifuge at 4000 rpm for 4 minutes and discard the supernatant (Aliquot 2). Repeat once.

3. Wash the column with 10 ml Nuclease Digestion Buffer (40 mM Tris / 6 mM $MgCl_2$, pH 7.5) Centrifuge at 4000 rpm for 4 minutes and discard the supernatant. Repeat two times.

4. Incubate with 250 U/ml DNase I (Amersham Biosciences) in 3 ml Nuclease Digestion Buffer at 37°C at rotation for 1 hour. Centrifuge at 4000 rpm for 4 minutes and discard the supernatant. (Aliquot 3)

5. Wash the column with 10 ml Buffer A. Centrifuge at 4000 rpm for 4 minutes and discard the supernatant (Aliquot 4) Repeat once.

6. Elute the antibodies with 4 ml 0,1M citric acid pH 3.0. Centrifuge at 4000 rpm for 5 minutes at 4°C.

7. Collect the supernatant, and immediately add 0.5 M Tris to neutralize the solution to approximately pH 7.5 (Aliquot 5).

Direct (and indirect) immunofluorescence microscopy (DIF)

1. Fresh cut cryosections (4μm) were transferred to super-frost slides, and allowed to dry in room temperature for 15-20 minutes.

2. The sections were incubated with 70 μl rat anti-mouse C1q (Cellsciences) diluted 1:50 in PBS with 10% goat serum and 1% BSA for 30 minutes in a moisture chamber at room temperature.

3. Gently aspirate the primary antibody and wash by gently sequential dipping the sections in PBS five times to remove unbound antibody.

4. Prepare the secondary antibody solution by diluting the fluorochrome labelled antibodies in PBS with 10% goat serum and 1% BSA: AlexaFluor 488- conjugated donkey anti-rat (Molecular Probes), and AlexaFluor 568- conjugated goat anti-mouse (Molecular Probes), both diluted 1:500.

5. Add 70 μl of the secondary antibody dilution and incubate the sections 30 minutes in a moisture chamber in room temperature and protected from light.

6. Wash with PBS five times to remove unbound antibody.

7. The sections were mounted under a coverglass with a drop of PBS and seal around the edges with transparent nail polish.

8. The sections were immediately investigated in fluorescent microscope (BX51, Olympus), and images were processed using Cell ^F soft imaging system from the same manufacturer

Immune Electron Microscopy (IEM) and Co-localization IEM (CIEM)

Handlings of the grids were done by a wire loop. The incubations were done on drops of liquids and the specimen side of the grid should be floating in contact with the fluid. The grids were careful lifted from one bath of reagent to the next, including 1x PBS for washing. The baths differed in volume according to different reagents, but all were drops of liquid on a piece of parafilm. The procedure was performed in room temperature. To avoid evaporating, the 15 minutes incubations were done in a moisture chamber, i.e. pre-wet filter paper covered by an overturned petri-dish (Webster&Webster)

1. Transfer the grids to drops of MilliQ water and leave it for 6.5 minutes. Repeat this two more times.
2. Incubate the grids in a drop of 1% fish skin gelatine (FSG) (Sigma-Aldrich) for 15 minutes to block for unspecific epitopes.
3. Rabbit anti-mouse (ICN/Cappel) was diluted 1:150 in PBS/FSG, and each grid was incubated in a 5 µl drop for 20 minutes (Detection of injected antibodies)
4. Wash the grid by transferring to large drops of PBS and leave it for two minutes. Repeat this four more times.
5. Protein A conjugated with 5nm gold particles (PAG-5) is diluted in FSG. The dilution is according to the manufacturer recommendations for the actual batch. Each grid is incubated in 5 µl drops for 15 minutes. (Visualizing the binding)

If co-localization is performed:

6. Incubate the grids in drops of 1% glutaraldehyde. Cover with petri dish because of potential health damage. (Blocking unspecific epitopes/ stabilize proteins)
7. Incubate the grids in drops of 0.12% glycine in PBS for 5 minutes. (Saturation of free aldehyde groups.)
8. Wash each grid by transferring to large drops of PBS and leave it for two minutes. Repeat this four more times.
9. Mouse anti-dsDNA antibody, 163p77, (provided by Tony Marion) was diluted 1:360 PBS/FSG, and each grid was incubated in a 10µl drop for 20 minutes. (Detection of DNA)
10. Rabbit anti-mouse (ICN/Cappel) was diluted 1:150 in PBS/FSG, and each grid were incubated in a 10µl drop for 2 minutes (Bridge)
11. Wash each grid by transferring to large drops of PBS and leave it for two minutes. Repeat this four more times.

12. Protein A conjugated with 10nm gold particles (PAG-10) are diluted in FSG. The dilution is according to the manufacturer recommendations for the actual batch. Each grid is incubated in 5 µl drops for 15 minutes. (Visualizing the binding)

13. Wash each grid by transferring to large drops of PBS and leave it for two minutes. Repeat this four more times.

14. Wash each grid by transferring to large drops of MilliQ water and leave it for 2.5 minutes. Repeat three times.

15. Methylcellulose (2%) and uranylacetate (3%) is mixed 1:10 and 3 drops are applied to a petri dish on ice with bottom covered with parafilm. The grids are consecutively dipped in the first two drops and left for 6 minutes in the third. (Contrast) Cover the petri dish because of potential health damage.

16. The grids are left to dry in room temperature for 20 minutes.

17. Examine the sections on the grids with transmission electron microscope (Jeol 1010) (25-50k total magnification)

7.2 Instruments, equipment, reagents

Cell culture

	Type / Description	Producer / Supplier
Equipments	Tissue culture flask: T25, T75 (cm2)	Nunc, Denmark
	CELLine 1000	BD Biosciences, cat.no 353137
	Pipettes: 5 ml, 10 ml	Falcon, Becton Dickinson Labware
	Hemocytometer: 1 mm	Burker
Medium	RPMI 1640	Sigma, cat.no R-6504
	FBS	PAA laboratories GmbH, Pasching, A15-043
	L- glutamine	Sigma, cat.no G-3126
	Interleukin- 6	Sigma, cat.no I-9646
Instruments	Laminar flow cabinet: V4II, ASSAB	Kebo, Sweden
	Incubator: Water Jacketed CO2	Forma Scientific
	Light Microscope: Leitz, DM IL	Leica
	Centrifuge: Minor	MSE

Purification / Dialyzing / Concentration

	Type / Description	Producer / Supplier
Equipments	Column	Pierce, USA
	Dialysis cassettes: Slide-A-Lyzer	Pierce, USA, cat.no 66810
	Dialysis tubes: 12-14 kD	Visking, Medicell, USA
Reagents	Protein A-Sepharose 4B Fast Flow	Sigma, cat.no. P-9424
	DNase I, FPLCpure	Amersham Biosciences, cat.no. 27-0514-02
	Polyethylene glycol (PEG 35000)	Fluka, cat.no. 81310
Instruments	Centrifuge: Megafuge 1.0	Heraeus, Houm AS, Norway
	Spectrophotometer: ND-1000	Nanodrop Technologies Inc, DE, USA

Injection / sampling

	Type / Description	Producer / Supplier
Equipments	Desalt column	Pierce
Antibody	Polyclonal Mouse IgG,	Caltag, Invitrogen, cat.no. 10400
Reagents	No-weight ^{TM} Sulfo-NHS-LC-biotin	Pierce
	Uristix	Bayer Diagnostics, Bridgend, UK
	OCT compound	Tissue Tek, Sakura Finetek, CA. USA
	2-Methylbutane Isopentan	Fluka, Sigma-Aldrich, cat.no.59070
	Paraformaldehyde	Merck 1.1.04005.1000
	McDowells fixative*	
Instruments	Centrifuge: Biofuge 13R	Heraeus

* McDowell, E.M. 1976 and Trump B.F. Histologic Fixatives Suitable for Diagnostic Light and Electron Microscopy Arch Patjol Lab Med- vol 100, aug.

ELISA

	Type / Description	Producer / Supplier
Equipments	Microtiter plates: MaxiSorp, 96 well	NUNC, Copenhagen, Denmark
	Adhesive plate seal	ABgene, cat.no. AB-0580, UK
Antigens	Calf thymus DNA, 10mg/ml	Sigma, cat.no D-8661
	Phosphatidylserine 10mg/ml	Sigma, cat.no. P-6641
	β2-Glycoprotein-1, 4,3 mg/ml	Chrystal Chem Inc. IL 60515 USA
	Phosphatidylcholine, 100mg/ml	Fluka, cat.no.61752
	Matrigel matrix, 10,3mg/ml	BD Biosciences, cat.no 356237
Antibodies	Anti-mouse IgG, γ-chain specific, HRP	Sigma, cat no. A-3673
	Anti-Phosphatidylserine, clone 1H6	Upstate, CA, cat no. 05-719
	Anti-rabbit IgG, HRP	DAKO P217
	Anti-laminin, 0,6mg/ml	Sigma, cat.no L-9393
	Anti-biotin	Roche
Reagents	O-phenylenediamine dihydrochloride	Sigma, cat.no P-8787
	Hydrogenperoxide 30%	Hospital Pharmacia, Tromsø
	Polyoxyethylene sorbitol monolaurate(Tween 20)	Sigma, cat.no. P-1379
Instruments	Incubator: B8000	Termaks
	Well washer: Wellwash 4	Labsystems
	Sonicator: Sonifier B-12	Branson sonic power comp. Con. USA
	ELISA reader: Multiscan Ascent	Thermo

Agarosegel electrophoresis

	Type / Description	Producer / Supplier
Equipment	Electrophoresis: Wide mini sub cell system	Bio-Rad, USA
Reagents	SeaKem LE agarose	BioWhittaker, MedProbe
	MW-standard: TriDye 1kb DNA ladder	BioLabs
	Ethidium bromide solution	Fluka
Instrument	Photosystem: GelDoc 2000	Bio-Rad, CA, USA

SDS-PAGE

	Type / Description	Producer / Supplier
Equipment	Electrophoresis: Xcell SureLock Mini-cell	Invitrogen , USA
Reagents	10 % 4-12 % Bis-Tris gel, NuPAGE Novex	
	MES SDS Running Buffer*	
	LDS Sample Buffer x 4	
	Dithiothreitol (DTT) : Reducing Agent x 10	
	MW-standard: SeeBluePlus2	
	Coomassie SimplyBlue SafeStain	
Instrument	Photosystem: GelDoc 2000	Bio-Rad, CA, USA

*MES running buffer (50 mM Tris base, 50 mM 3–(N–Morpholino) propanesulfonic acid, 1 mM EDTA, and 0.01% SDS at pH 7.3)

Biacore

	Type / Description	Producer / Supplier
Biotinylation	Mouse genomic DNA	Calbiochem, Germany
	Biotin-16-dUTP	Roche, Switzerland
	Nick translation kit	Vysis, Abbott molecular Inc. IL, USA
Equipment	Sensor chip SA	Biacore, Uppsala, Sweden
Solutions	Running buffer: 10 x HBS-EP+	
	BIAnormalizing solution	
	Regeneration solution: NaOH 50 (50mM)	
Instrument	Biacore T100	

DIF

	Type / Description	Producer / Supplier
Equipment	Microscope slides: SuperFrost Plus	Menzel GmbH & Co, J1800AMNZ
Antibodies	Anti-Mouse C1q (Rat)	Cell Sciences Inc. MA, cat.no HM 1044
	Donkey anti-rat IgG, AlexaFluor 488	Molecular Probes, cat.no A-21208
	Goat anti-mouse IgG, AlexaFluor 568	Molecular Probes, cat.no A-11031
Instruments	Microtome: Kryostat 1720	Leitz
	Fluorescence microscope: BX51,O-RFL-T,	Olympus
	Image processing: Cell ^F soft imaging system,	Olympus

IEM

	Type / Description	Producer / Supplier
Antibody	Rabbit-anti mouse IgG	ICN /Cappel, Aurora, OH
Reagents	Fish skin gelatine	Sigma-Aldrich, St.Louis, MO
	Protein A-gold, 5nm and 10nm,	University of Utrecht, The Netherlands
	Glutaraldehyde,	Merck 1.04239.1000
	Glycine	Sigma G7126
	Uranylacetate	Fluka 73945
	Methylcellulose	Sigma M-6385
Instruments	Transmission electron microscope: JEM-1010	Jeol, Tokyo, Japan
	Morada 11M pixels digital camera Image processing: iTEM	Olympus Soft Imaging System, Münster, Germany