



Nucleosomes and C1q bound to glomerular endothelial cells serve as targets for autoantibodies and determine complement activation

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ABSTRACT

Various studies indicate a role for both anti-nucleosome and anti-C1q autoantibodies in glomerulonephritis in patients with systemic lupus erythematosus. However, a causal relationship between these autoantibodies and the development of lupus nephritis has not been fully established. Since injury of the endothelium is a major target in lupus nephritis we assessed the interaction of C1q and nucleosomes with glomerular endothelial cells *in vitro* in the presence or absence of autoantibodies against these antigens. We demonstrate a direct and dose-dependent binding of both nucleosomes and C1q to immortalized human glomerular endothelial cells (GENC) *in vitro*, which in part is mediated by cell surface heparan sulfate. We demonstrate that nucleosomes and C1q serve as targets for monoclonal and polyclonal antibodies as well as for anti-nuclear autoantibodies from patients with systemic lupus erythematosus. An additive effect of anti-C1q autoantibodies on anti-nucleosome mediated complement activation was observed. Furthermore, we showed that the activation of complement on glomerular endothelial cells is mediated by the classical pathway since the deposition of C3 on GENC is abrogated by MgEGTA and does not occur in C1q-depleted serum. Taken together, our studies demonstrate a direct binding of both nucleosomes and C1q to glomerular endothelial cells *in vitro*. The subsequent binding of autoantibodies against nucleosomes in patients with systemic lupus erythematosus is potentially pathogenic and autoantibodies against C1q seem to have an additional effect.

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1. Introduction

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease characterized by B cell hyperactivity, production of a multitude of different autoantibodies directed primarily against nuclear antigens, such as nucleosomes, and immune complex formation. The initial triggers that lead to the autoimmune response in SLE may be explained by a reduced clearance of apoptotic material and/or an aberrant apoptotic process (Munoz et al., 2008). SLE affects about 0.04% of the general population in developed countries. Nearly 80% of the cases occur in women in the childbearing years, and it may affect as many as 1 in 1000 young women (Mills, 1994). The etiology

of SLE is largely unknown, however it involves genetic, hormonal, and environmental factors (Tsao, 2003).

Antibodies against double-stranded DNA (dsDNA) are regarded as highly specific for the disease (Berden, 1997) and anti-dsDNA antibodies are found in the majority of SLE patients (Grootscholten et al., 2007). It is established now that the nucleosome is the driving autoantigen in SLE (Dieker et al., 2002) and it has been suggested that the presence of anti-nucleosome antibodies could serve as a better marker for SLE than anti-dsDNA antibodies since anti-nucleosome antibodies are present in up to 90% of lupus patients (Cervera et al., 2003; Bruns et al., 2000). In SLE many organs may be affected, including serosa, joints, CNS, skin, and kidney. Lupus nephritis (LN) is found in approximately 25–50% of SLE patients (Cameron, 1999) and is the major cause of morbidity and mortality (Davis et al., 1996). LN is associated with a rise in titer of especially high avidity anti-dsDNA IgG and reduced levels of serum complement. Deposited immune complexes in the glomeruli induce complement activation and are involved in the pathogenesis of lupus nephritis (Bruns et al., 2000; ter Borg et al., 1990). Moreover, elution studies of glomeruli of SLE patients and lupus mice have revealed the presence of anti-dsDNA, anti-histone and anti-nucleosome

Abbreviations: GENC, glomerular endothelial cell; HS, heparan sulfate; HUVS, hypocomplementemic urticarial vasculitis; LN, lupus nephritis; PHS, pooled human serum; SLE, systemic lupus erythematosus.

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antibodies, and their respective antigens (van Bruggen et al., 1997a, 1997b; Kramers et al., 1996). The molecular mechanisms of how these antibodies in conjunction with their antigen activate complement within the glomerulus, however, remains unclear.

As mentioned, the complement system plays an important role in the onset as well as the effector phase of LN in SLE (Berden, 1997; Eiser et al., 1979). Complement itself may also be the target of an autoantibody response (Seelen et al., 2003).

Next to anti-dsDNA antibodies, anti-C1q autoantibodies have been found to be closely associated with LN (Siebert et al., 1991). There is evidence that a rise in anti-C1q autoantibody titers may predict flares of nephritis (Siebert et al., 1991; Coremans et al., 1995). Additionally there is an accumulation of anti-C1q autoantibodies in kidneys from patients with SLE and renal involvement (Mannik and Wener, 1997; Mannik et al., 2003). Conversely, in the absence of anti-C1q autoantibodies, signs of LN seem to be absent (Fremeaux-Bacchi et al., 2002). Interestingly, anti-C1q autoantibodies can be found in several other disease conditions, such as hypocomplementemic urticarial vasculitis (HUVS), and even in some healthy individuals (Kohro-Kawata et al., 2002; Seelen et al., 2003), but in these instances anti-C1q antibodies are not related to renal pathology (Siebert et al., 1992).

Anti-C1q autoantibodies also occur in murine models of SLE (Hogarth et al., 1996; Trinder et al., 1995; Trouw et al., 2004a). In MRL/lpr mice, a rising anti-C1q autoantibody titer is observed in parallel with an increase in renal injury. In order to obtain insight in the possible causal relationship between anti-C1q antibodies and renal injury, Trouw et al. (2004a) injected naïve mice with mouse monoclonal and rabbit polyclonal antibodies directed against C1q, which induced significant renal inflammation, as assessed by the glomerular infiltration of polymorphonuclear leucocytes. However, a full blown renal injury in the form of proteinuria was not observed suggesting that additional triggers are required for significant renal damage.

Indeed, combination of anti-C1q antibodies with a sub-threshold dose of anti-glomerular basement membrane antibodies led to significant proteinuria. Since both anti-C1q and anti-dsDNA antibodies seem to be involved in LN, we isolated nucleosomes, which are the main target of anti-DNA and anti-nucleosome antibodies in the kidney of human as well as murine SLE (Rekviq and Nossent, 2003; van Bruggen et al., 1996, 1997a, 1997b), and found that they bind directly to glomerular endothelial cells (GEnC), independently of antibodies. Furthermore, we assessed the effects of anti-nucleosome and anti-C1q autoantibodies from patients with SLE versus that of healthy controls on complement activation using conditionally immortalized glomerular endothelial cells (GEnC) *in vitro*.

2. Material and methods

2.1. Generation of nucleosomes and interaction with C1q

Jurkat cells were cultured in standard culture medium (RPMI 1640, Invitrogen, Breda, the Netherlands) supplemented with 10% of fetal calf serum and penicillin/streptomycin. Apoptosis was induced with ultra-violet irradiation of 100 J/m² and the cells were subsequently cultured under serum-free conditions for another 10 days. Supernatants from these apoptotic cell cultures were used for the isolation of DNA using the phenol, chloroform and isoamyl alcohol extraction method. Nucleosomes in the culture supernatant were further purified by size exclusion chromatography on Sepharose S200 (Pharmacia), and fractions were collected and subsequently coated on an ELISA plates (NUNC, Roskilde, Denmark) (Kramers et al., 1994) and assessed for reactivity with a specific anti-nucleosome monoclonal antibody. Fractions containing nucle-

osomes were pooled and analyzed for nucleosome components and reactivity with monoclonal antibodies against DNA (moAb #36), histones (moAb #34) and nucleosomes (moAb #32) (Dieker et al., 2005).

DNA and histones were commercially obtained (Invitrogen, Breda, the Netherlands), respectively (Roche, Mannheim, Germany). Binding of C1q to nucleosomes was evaluated on coated nucleosomes in an ELISA plate (NUNC) and subsequent incubation with increasing concentrations of purified human C1q, in the presence or absence of potential inhibitors (BSA, DNA or nucleosomes), was performed in PBS with BSA (1%) and Tween-20 (0.02%), abbreviated as PBS-BSA-Tween. Bound C1q was detected with a mouse monoclonal antibody against human C1q and a goat anti-mouse IgG conjugated to horse radish peroxidase (HRP) (DAKO, Glostrup, Denmark) and 3,3',5,5'-tetramethylbenzidine (ABTS). Data are expressed as optical density values at 415 nm (OD415).

2.2. Glomerular endothelial cells (GEnC)

Conditionally immortalized human GEnC have been described in detail by Satchell et al. (2006). Briefly, the GEnC were cultured in 96 wells plates (Costar), allowed to proliferate at a permissive temperature of 33 °C, until confluent, and, subsequently, cultured at a non-permissive temperature of 37 °C for, at least, 5 days. GEnC were cultured in EGM-2/bullet kit medium (Lonza, Breda, the Netherlands), containing VEGF and other growth factors. In some experiments, heparinase III (Sigma-Aldrich, Zwijndrecht, the Netherlands), diluted in culture medium at a concentration of 0.25 U/ml for 60 min at 37 °C, was used to remove heparan sulfate chains from GEnC as was described before (Lehtinen et al., 2009; Rops et al., 2007, 2008).

After culture or incubation with different reagents, GEnC were washed three times with PBS-BSA-azide. GEnC were subsequently incubated with purified Jurkat-derived nucleosomes (0–800 ng/ml) and/or purified human C1q (0–100 µg/ml) diluted in culture medium for 30 min at 4 °C. C1q was purified as described previously (Oroszlan et al., 2007). Human serum samples from either healthy controls or patients with systemic lupus erythematosus were heat-inactivated for 30 min at 56 °C and diluted (1:2 and 1:4) in culture medium. Before addition to cell cultures, normal human serum was used as a source of complement at a concentration of 30% in culture medium containing 5 mM MgCl₂ and CaCl₂ for complement activation which was then incubated at 37 °C. In some experiments 5 mM MgEGTA was added to block the classical and lectin pathway of complement. C1q depleted human serum (Nauta et al., 2002) was also used to prevent classical pathway of complement activation. After incubation with the complement source, cold PBS-BSA was added to stop the activation of complement. After incubation with the required agents adherent GEnC were detached with PBS-BSA-azide containing lidocaine (5 mg/ml) for 30 min at 37 °C. Detached GEnC were then incubated in PBS-BSA-azide with detecting antibodies. For the detection of GEnC-bound nucleosomes, a monoclonal mouse anti-nucleosome IgG (#32) was used. For the detection of GEnC-bound C1q, a monoclonal mouse anti-human C1q IgG (Nauta et al., 2002) was employed and for the detection of human C3, a monoclonal mouse anti-human C3 (RFK22) (Nauta et al., 2003) was used. For the detection of human IgG, a monoclonal mouse anti-human IgG (American Type Tissue Culture Collection, HB43) was used. Goat anti-mouse allophycocyanin (APC) (DAKO) was used to detect the binding of these monoclonal mouse antibodies. Polyclonal digoxigenin (DIG)-conjugated rabbit (generated in our laboratory) anti-human C1q antibodies and goat anti-DIG FITC-conjugated antibodies (Roche) were applied to detect binding of human C1q to GEnC (van den Berg et al., 1998). Cells were analyzed by fluorescent activating cell sorting (FACS) on

a Becton Dickinson FACS Calibur flowcytometer, using CellQuest software.

3. Results

3.1. Isolation and characterization of nucleosomes from apoptotic Jurkat cells

Nucleosomes of human origin were generated from apoptotic Jurkat cells. DNA extracted from nucleosomes released into the supernatant of apoptotic Jurkat cell cultures typically exhibited bands of approximately 150, 300 and 450 bp (Fig. 1a), indicating that mononucleosomes, dimers and trimers of nucleosomes (300 and 450 bp) were released in the supernatant. To show that nucleosomes were generated with this method, the pool of nucleosomes after size exclusion chromatography on Sepharose S200 were coated in 96-well plates and assessed for reactivity with a mouse anti-nucleosome monoclonal antibody. In addition DNA and histones were used as a coating, and the reactivity of anti-DNA and anti-histone antibodies were tested in ELISA as well (Fig. 1b). The mouse anti-nucleosome monoclonal antibody only recognizes intact nucleosomes and not DNA and or histones indicating that we obtained intact nucleosomes.

3.2. Binding of nucleosomes to human glomerular endothelial cells

To determine whether nucleosomes are able to interact with human glomerular endothelial cells and thereby potentially serve as a target for anti-chromatin autoantibodies, we made use of a

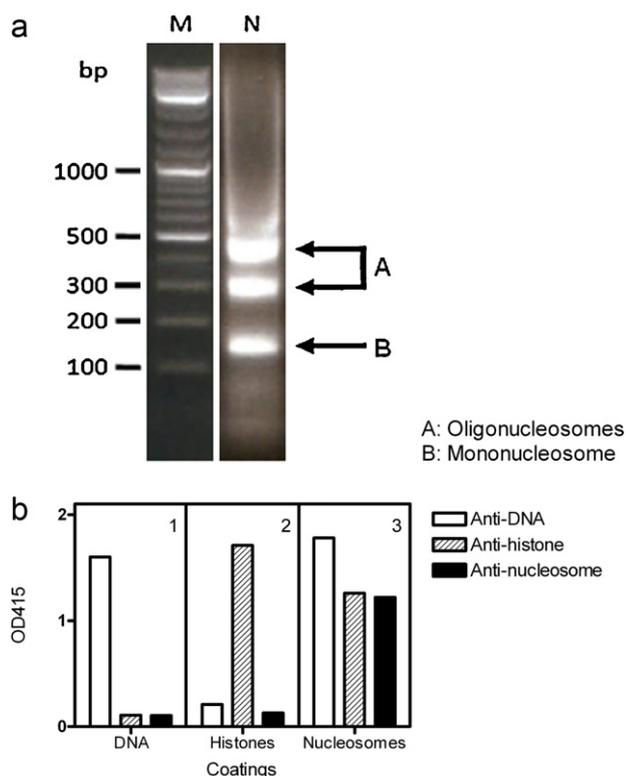


Fig. 1. Isolation and characterization of nucleosomes. SDS PAGE analysis of Sepharose S200 purified nucleosomes (panel (a)). Lane M contains a molecular weight marker and lane N consists of purified nucleosomes. Panel (b) shows the reactivity of specific monoclonal antibodies against DNA (open bars), histones (hatched bars) and nucleosomes (dark bars) with the purified oligonucleosomes, DNA and histones in ELISA. The ELISA plates in (b) panels 1, 2 and 3 were coated with DNA, histones or with nucleosomes, respectively.

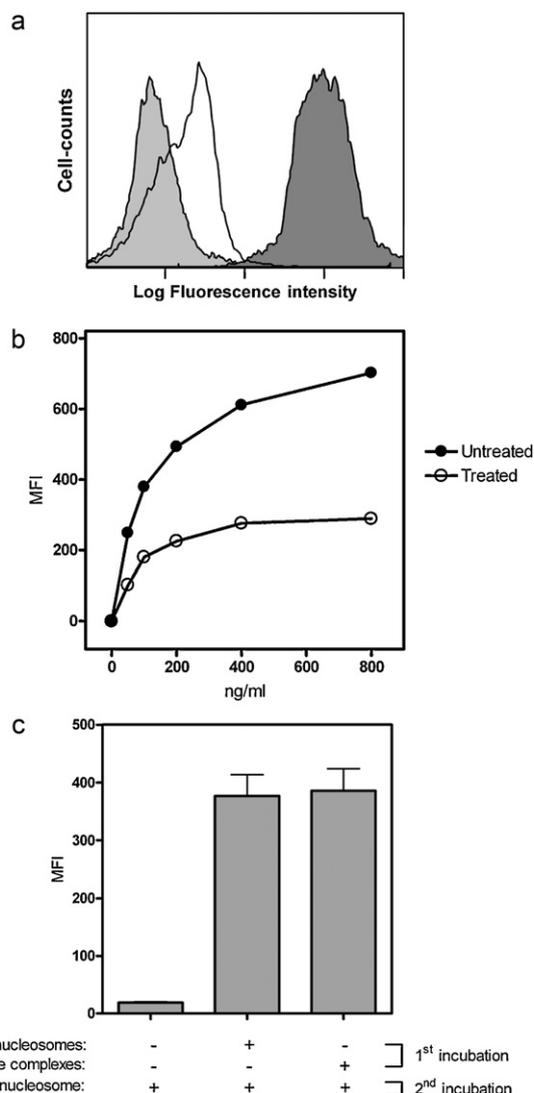


Fig. 2. Binding of nucleosomes to glomerular endothelial cells. Binding of nucleosomes to GENC: GENC in culture wells were incubated with nucleosomes, or medium alone, washed and subsequently incubated with a control monoclonal antibody or with a anti-nucleosome monoclonal antibody, washed and bound antibody was detected with goat anti-mouse allophycocyanin as described in Section 2. Panel (a) shows that there is significant binding of nucleosomes to GENC (dark grey profile) as compared to the control antibody (open profile) and the cells exposed to the goat antibody alone (grey profile). The binding of nucleosomes to GENC is dose dependent (panel (b), closed symbols) and is reduced when the GENC are pretreated with heparinase (panel (b), open symbols). In panel (c) the results are shown of the binding of nucleosomes alone or nucleosomes in immune complex form to GENC as detected with an anti nucleosome antibody. The results are the mean \pm SD of 4 separate determinations.

conditionally immortalized glomerular endothelial cell line (GENC) (Satchell et al., 2006). Exposure of GENC to nucleosomes followed by FACS analysis, revealed significant and direct binding of nucleosomes (Fig. 2a). Cells not exposed to nucleosomes (control antibody) have a low mean fluorescent index, whereas the monoclonal antibody control without nucleosomes shows some background staining. To demonstrate the possible involvement of heparan sulfate (HS) in the binding of nucleosomes to GENC, adherent cells were pre-treated with heparinase III or left untreated. Subsequently, the cells were incubated with increasing concentrations of nucleosomes, and analyzed by FACS. The binding of nucleosomes to GENC was dose-dependent and the binding was significantly reduced following heparinase III pretreatment of the GENC by approximately 60% at all concentrations of nucleosomes,

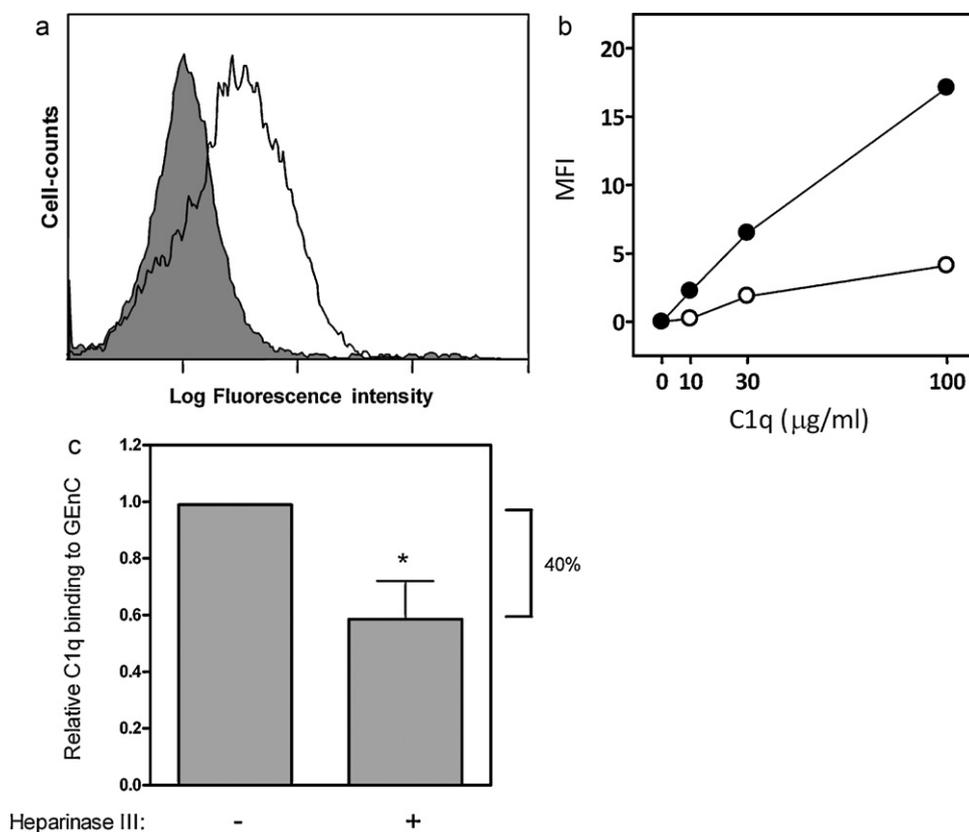


Fig. 3. Binding of C1q to glomerular endothelial cells. GEnC in culture wells were exposed to 100 µg/ml of C1q or BSA, washed and then bound C1q was detected using a mouse monoclonal antibody against C1q (dark line) or a control monoclonal antibody (grey profile) as described under Section 2 (panel (a)). The binding of C1q to GEnC was dose-dependent (panel (b), closed symbols) and was reduced when the GEnC were pre-treated with heparinase (panel (b), open symbols). In panel (c) the binding of a fixed concentration of C1q (10 µg/ml) to GEnC either pretreated with heparinase or not is depicted for an experiment performed in triplicate (* $p < 0.05$).

indicating that the binding of nucleosomes to GEnC is, at least in part mediated via heparan sulfate structures on GEnC (Fig. 2b). From the literature, it is known that nucleosome/anti-nucleosome immune complexes (IC), but not anti-nucleosome antibodies alone, are able to react with glomeruli of rats *in vivo* (Kramers et al., 1994). Therefore, GEnC were incubated with immune complexes containing nucleosomes and mouse anti-nucleosome IgG2a as compared to nucleosomes alone for 30 min at 37 °C. The presence of mouse antibodies (as a measure of bound IC) was readily detectable by FACS, demonstrating that immune complexes bind to GEnC (Fig. 2c), most likely via the interaction between highly positively charged histones within the nucleosome and negatively charged HS structures. To find out whether antibody-mediated binding to Fc receptors for IgG isotypes (FcγRs) could be involved in the binding of immune complexes to GEnC, we assessed the expression of FcγRs on GEnC. No detectable expression of FcγRs on GEnC was observed, while peripheral blood-derived cells from a healthy donor were clearly positive (data not shown). Furthermore, when aggregated human IgG was used as a model for immune complexes, no binding of these aggregates to GEnC was observed (data not shown). Taken together, these data indicate that nucleosomes bind directly to GEnC, and not as a result of antibody-dependent mechanisms.

3.3. C1q binds to human glomerular endothelial cells

It has been described that C1q can directly bind to human umbilical vein endothelial cells (Daha et al., 1988; van den Berg et al., 1998). Therefore, we tested the ability of GEnC for the binding of C1q as well (Fig. 3). Detached GEnC were incubated with purified human C1q, and bound C1q was detected by a monoclonal mouse

anti-human C1q and analyzed by FACS. As depicted in Fig. 3a a clear binding of C1q to GEnC occurs. This binding is specific and dose-dependent (Fig. 3b). Also in this case we evaluated the possible role of HS in mediating direct binding of C1q to GEnC. Removal of HS resulted in a decreased binding of C1q to GEnC (Fig. 3b and c), which contributed to about 40% of C1q binding (Fig. 3c). In summary, C1q can bind directly to GEnC, which in part is mediated by cell surface HS.

3.4. Binding of C1q to nucleosomes

It has been described that C1q serves as a soluble pattern recognition molecule for apoptotic cell debris and that it is implicated in the recognition of lupus antigens, including nucleosomes. Exposure of these self-antigens to the acquired immune system may lead to the generation of autoantibodies. In this context C1q may serve as a target for autoantibodies in SLE as well. It has been suggested that C1q bound to apoptotic cells for example via its interaction with nucleosomes leads to the exposure of neo-epitopes that are recognized by anti-C1q autoantibodies in patients with SLE (Flierman and Daha, 2007). To investigate whether C1q is able to bind to nucleosomes, nucleosomes were coated in 96-well plates and, subsequently, incubated with increasing concentrations of C1q. Bound C1q was detected with a monoclonal mouse anti-human C1q (Fig. 4a). A dose-dependent binding of C1q to nucleosomes was observed. When a fixed amount of C1q (10 µg/ml) was incubated in the presence of increasing concentrations of potential inhibitors such as DNA and nucleosomes, a dose-dependent and decreased binding of C1q to coated nucleosomes was observed (Fig. 4b), indicating that C1q binds, at least in part, to nucleosomes via their DNA content. A role for the globular heads of C1q in the recognition is

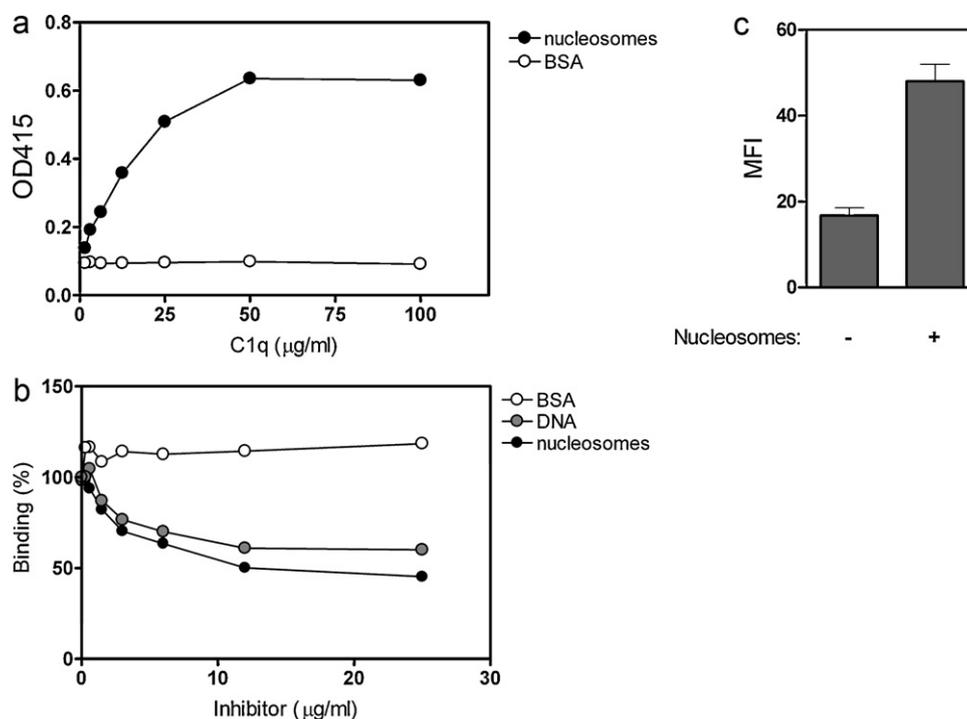


Fig. 4. *In vitro* interactions of C1q with nucleosomes. Panel (a), ELISA wells were coated with increasing concentrations of nucleosomes or BSA, washed and then reacted with a fixed concentration of C1q, washed and bound C1q detected with monoclonal antibody against C1q. In panel (b) it is shown that the binding of nucleosomes (coated) to C1q (10 μg/ml) is inhibited in a dose dependent fashion by nucleosomes and DNA but not by BSA. In panel (c), the results of binding of C1q to GEnC are shown. GEnC in culture were exposed to BSA or to 10 μg/ml of nucleosomes in triplicate wells, washed and then assessed for binding of C1q (100 μg/ml) by FACS as described in Section 2. Results are the mean ± 1 SD.

suggested as the ability of C1q to recognize nucleosomes in ELISA was abrogated after heat-inactivation of C1q.

As binding of C1q to GEnC also was seen in the absence of nucleosomes (see Fig. 3a–c), we wondered whether the binding of C1q to GEnC could be enhanced after pre-incubation of these cells with nucleosomes. Indeed, the binding of C1q is dramatically enhanced when nucleosomes are already bound to GEnC (Fig. 4c). Notably, the enhancing effect of nucleosomes in binding of C1q to GEnC overrides the contributions of HS mediated binding of nucleosomes and C1q. These findings strongly suggest that C1q binds to nucleosomes on the surface of GEnC and, thereby, is a potential target for anti-C1q autoantibodies.

3.5. Anti-nucleosome and anti-C1q autoantibodies lead to complement activation on GEnC

In order to obtain insight in the possible injurious effects of autoantibodies from sera of SLE patients on complement activation, GEnC were sensitized with C1q alone, nucleosomes alone, the combination of both agents or BSA as a control, washed and then incubated with two-fold dilutions of heat inactivated pooled human serum (PHS), or heat inactivated serum of a patient containing both anti-C1q and anti-DNA autoantibodies (see experimental design in Fig. 6a). Thereafter the cells were washed and incubated with a fixed dilution (30%) of normal human serum (non heat treated) as a source of complement. The degree of C3 deposition, as a measure for complement activation, was then quantified by FACS as described previously (Gaarkeuken et al., 2008).

BSA-sensitized GEnC incubated with dilutions of NHS as a complement source showed only background C3 deposition. C1q treated GEnC also exhibited minimal amounts of complement activation that was not distinguishable from the BSA controls (Fig. 5b). Nucleosome-sensitized GEnC induced a significant increase in C3

deposition and the double sensitization with C1q and nucleosomes further increased the amount of C3 deposition on the GEnC.

The effect of serum of an SLE patient containing both anti-C1q and anti-DNA on BSA-sensitized GEnC was minimal and induced limited C3 deposition on the GEnC (Fig. 5c). The effect of SLE serum on C1q-sensitized GEnC was also limited as compared to the C1q/nucleosome (double) sensitized cells; however, there was significant C3 deposition as compared to the BSA controls. Nucleosome sensitized GEnC exposed to SLE serum was significantly enhanced and dependent on the dilution of the autoimmune serum reaching plateau levels at dilutions of 1:4 and 1:2.

In summary, the effect of autoimmune serum on GEnC sensitized both with C1q and nucleosomes in complement activation was superior to the effect of sensitization with C1q and nucleosomes alone.

To further explore the effects of serum containing only anti-DNA antibodies and the combined presence of both anti-C1q and anti-DNA on GEnC, a fixed dilution of 1:4 of heat-inactivated sera from normal donors ($n = 12$), SLE patients with anti-DNA alone ($n = 18$), and SLE patients having both anti-DNA and anti-C1q ($n = 21$) were tested. The experimental design is shown in Fig. 6a. The sera were tested on GEnC sensitized either with BSA alone, C1q alone, nucleosomes alone or the combination of C1q and nucleosomes, and finally assessed for C3 deposition (Fig. 6). GEnC sensitized with C1q and subsequently exposed to 12 normal sera did not support significant C3 activation as compared to the BSA controls. Nucleosome sensitized GEnC exposed to normal sera clearly supported complement activation, which was significantly higher than for the BSA and C1q sensitized GEnC. GEnC sensitized both with C1q and nucleosomes showed further enhanced C3 activation. These findings suggest that binding of nucleosomes to GEnC induces C3 activation on GEnC.

The second part of the experiment concerned the effects of SLE serum containing either anti-DNA alone or the combination of anti-

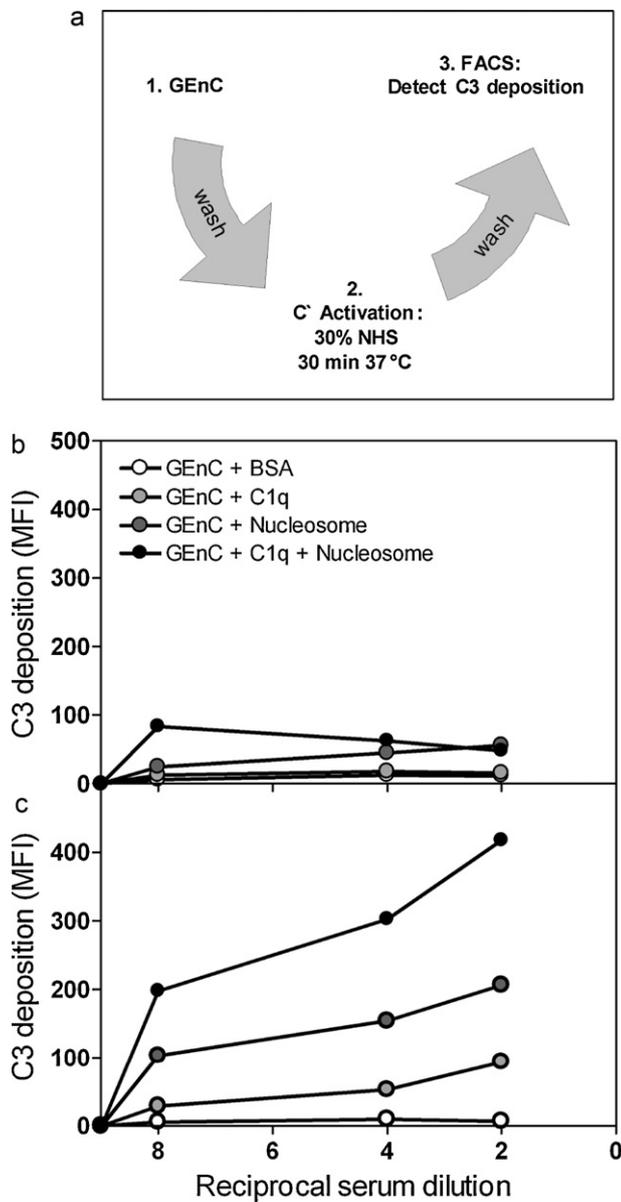


Fig. 5. Sensitization of glomerular endothelial cells with nucleosomes and C1q leads to complement activation after incubation with SLE serum containing autoantibodies. Effects of autoantibodies against DNA and C1q on GEnC sensitized with BSA, C1q, nucleosomes or the combination of C1q and nucleosomes. GEnC in culture wells, sensitized as indicated in panel (b) were first incubated with dilutions of heat inactivated pooled human serum and then assessed for their capacity to activate the complement system in 30% human serum (NHS) and then assessed for C3 deposition on GEnC by FACS (panel (a)). In panel (b) it is shown that GEnC sensitized with nucleosomes support complement activation and that sensitization with both C1q and nucleosomes leads to better complement activation while sensitization with BSA or C1q remains negligent. The experiment was performed twice and the result of one of the two experiments is depicted in (b). In panel (c) the cells sensitized as mentioned above were first exposed to dilutions of serum from a patient with SLE containing both anti-C1q and anti-DNA autoantibodies, washed and then further incubated with 30% human serum as a source of complement. It is interesting to note that GEnC sensitized with C1q and then reacted with autoantibodies (most probably anti-C1q) clearly support complement activation in a dose dependent fashion. In a similar way GEnC sensitized with nucleosomes dose dependently support complement activation. The degree of complement activation by GEnC sensitized both with C1q and nucleosomes is much stronger and seems to be at least additive to that of C1q and nucleosomes alone.

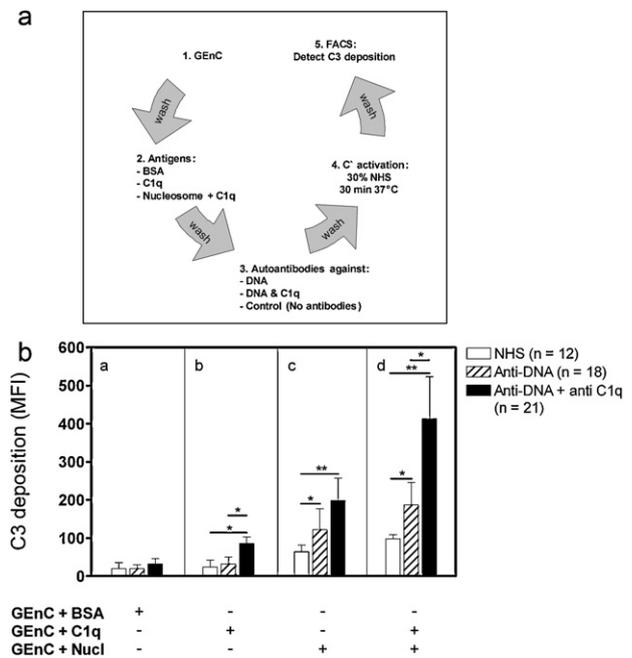


Fig. 6. Sensitization of glomerular endothelial cells with nucleosomes and C1q leads to complement activation after incubation with SLE sera containing anti-nucleosome and anti-C1q antibodies. GEnC in culture wells were first sensitized with BSA alone, C1q alone or with nucleosomes together with C1q, washed, and then reacted with sera containing no antibodies (control), anti-DNA antibodies or sera containing both anti C1q and anti-DNA. After washing, the cells were subjected to complement activation in 30% NHS and assessed for C3 deposition by FACS (a). (b) Activation of complement and subsequent deposition of C3 on GEnC sensitized with BSA alone (panel b–a), C1q alone (panel b–b), nucleosomes alone (panel b–c) and the combination of C1q and nucleosomes (panel b–d). The cells were then exposed to a fixed dilution of 1:4 of 12 sera from normal donors (open bars), sera from 18 SLE patients with anti-DNA alone (hatched bars) and 21 sera of SLE patients with both anti-C1q and anti-DNA (dark bars), washed and then incubated with 30% human serum as a complement source for 30 min at 37 °C and assessed for C3 deposition by FACS. The results are the mean \pm SD of C3 deposition. The horizontal bars indicate the *p*-value of difference **p* < 0.05; ***p* < 0.02.

DNA and anti-C1q. The reason for this choice being that there were no SLE sera available having anti-C1q alone. The effect of SLE sera on BSA sensitized GEnC were not different from that found in the normal control sera (Fig. 6b). C1q sensitization followed by exposure to anti-DNA containing sera alone did not result in an increase in subsequent C3 activation. Nucleosome sensitization alone and exposure to anti-DNA containing sera clearly resulted in subsequent C3 activation. The combination of sensitization of GEnC with both C1q and nucleosomes was significantly increased as compared to nucleosome sensitization alone (Fig. 6b).

Next the combined effects of anti-C1q and anti-DNA were analyzed. As before BSA sensitized GEnC and subsequent exposure of these cells to the SLE sera containing both anti-C1q and anti-DNA were not different from the results presented in Fig. 6b and c. The presence of anti-C1q resulted in a modest but significant increase in C3 deposition on the C1q sensitized GEnC, which is also clearly enhanced as compared to the effects seen with SLE sera that did not contain anti-C1q antibodies. The effects of anti-DNA together with anti-C1q on GEnC sensitized both with nucleosomes and C1q was significantly greater than the sum of C1q and nucleosome sensitization separately (Fig. 6b–d).

3.6. Pathway of complement activation

In order to obtain insight in the pathway of complement leading to C3 activation and C3 deposition on GEnC under different conditions of sensitization, GEnC sensitized with BSA alone, C1q alone,

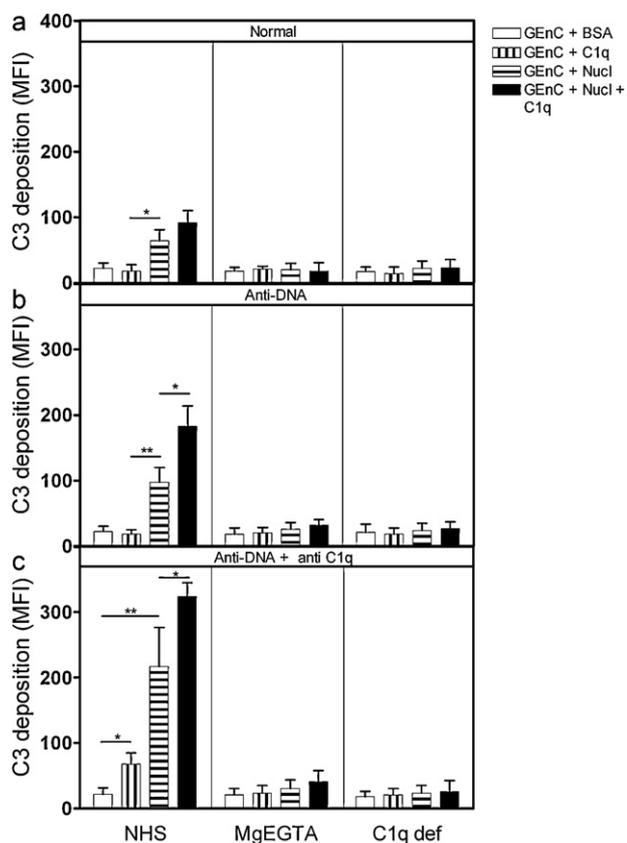


Fig. 7. Complement activation on glomerular endothelial cells sensitized with nucleosomes and C1q followed by incubation with SLE sera containing autoantibodies is mediated by the classical pathway. GEnC sensitized as indicated in Fig. 6 were washed and then incubated with a heat inactivated normal human serum, washed and then incubated with normal human serum (NHS) or with NHS containing MgEGTA or with C1q-deficient serum (panel (a)) for 30 min at 37 °C and assessed for C3 deposition by FACS. The results are the mean \pm 1 SD of triple determinations. In panels (b) and (c) the sensitized cells were sensitized as indicated, washed and then exposed to a fixed dilution of 1:4 of SLE serum containing only anti-DNA or containing both anti-C1q and anti-DNA. Thereafter the degree of C3 activation and deposition on the GEnC was quantified. The results indicate that under all the three conditions of exposure with antibodies classical pathway mediated C3 deposition occurs.

nucleosomes alone or the combination of the two latter agents, after washing, were exposed to 30% NHS as a complement source; 30% NHS containing 5 mM MgEGTA or 30% C1q-depleted serum, and assessed for C3 deposition on the GEnC.

As shown in Fig. 7a, BSA or C1q sensitized GEnC did not exhibit significant C3 deposition. Nucleosome sensitization resulted in clear C3 deposition on GEnC which was abrogated in the presence of MgEGTA and also did not occur in C1q-depleted serum. GEnC sensitized with both C1q and nucleosomes showed relatively more C3 deposition than GEnC sensitized with nucleosomes alone. Again this C3 deposition did not occur in MgEGTA or in C1q-depleted serum indicating activation of complement mainly via the classical pathway.

Next we explored the pathway of complement activation of the differently sensitized GEnC following treatment of the cells with an SLE serum containing only anti-DNA antibodies (Fig. 7b). Again BSA or C1q sensitized cells did not exhibit significant C3 deposition. Nucleosome sensitized GEnC and further opsonisation with anti-DNA showed clear C3 deposition which was absent in the presence of MgEGTA or in C1q-deficient serum. GEnC sensitized with both C1q and nucleosomes and further opsonised with anti-DNA exhibited a further increase in C3 deposition which did not occur in the presence of MgEGTA or in C1q-deficient serum.

Finally, the pathway of complement activation was assessed for the differently sensitized GEnC but now further opsonised with both anti-C1q and anti-DNA.

The results depicted in Fig. 7c show that the C1q opsonized GEnC also support C3 deposition and that this is also dependent on the classical pathway because the C3 deposition on the GEnC did not occur in the presence of MgEGTA or in C1q-deficient serum. In a similar way the pathway involved in complement activation was established for GEnC sensitized with nucleosomes or the combination of nucleosomes and C1q.

In summary, in all conditions where C3 deposition occurs a clear involvement of the classical pathway of complement activation is seen. The combined sensitization of GEnC by C1q and nucleosomes leads to the highest level of complement activation, which is mediated by the classical pathway.

4. Discussion

Several studies have shown a high association between the occurrence of anti-C1q autoantibodies and renal involvement in systemic lupus erythematosus (Coremans et al., 1995; Mannik and Wener, 1997; Siegert et al., 1991). Also the involvement of anti-DNA and or anti-nucleosome autoantibodies in the pathogenesis of renal disease in SLE has been implicated (Dieker et al., 2005; van Bavel et al., 2008). Experimental studies in mice and rats support both a role for anti-C1q antibodies and anti-DNA antibodies in renal injury, however, neither anti-C1q nor anti-DNA antibodies alone are able to induce significant renal inflammation resulting in significant proteinuria (Trouw et al., 2004b; Raats et al., 2000). Therefore, it has been postulated that for lupus nephritis more than one trigger is required for full blown renal disease.

In the present study we have explored the contribution of anti-C1q autoantibodies alone, the effect of anti-DNA alone or the combination of both, in an experimental *in vitro* setting to assess the potential injurious effects of these autoantibodies on glomerular endothelial cells. We have focused on the ability of these autoantibodies on complement activation because it has been shown that in SLE significant complement activation occurs both in the circulation as well as at the tissue level in the kidney itself.

To approach the questions above, immortalized GEnC were assessed directly for the binding of C1q and nucleosomes. We found a direct dose dependent binding of C1q to GEnC. This binding of C1q to GEnC occurs via the collagenous part of the molecule, as suggested by the inhibition of C1q binding by heat treatment of C1q. Part of the direct binding of C1q to GEnC is mediated by heparan sulfate at the cell surface. Subsequent incubation of the GEnC sensitized with C1q with human serum as a complement source did not support significant complement activation on the GEnC.

A very important observation in this study is that nucleosomes can bind directly and specifically to GEnC *in vitro*. Earlier *in vivo* studies in rats (Kramers et al., 1994; Schmiedeke et al., 1989) have suggested that trapping of nucleosomes in the glomerular basement membrane occurs when the nucleosomes are present in immune complex form. In the present study the binding of nucleosomes to cultured GEnC was assessed both using immune complexes and free nucleosomes. There was no significant difference between binding of nucleosomes in immune complex form or as free nucleosomes to GEnC. Previously we have shown that heparan sulfate is essential in the initial binding of nucleosome containing immune complexes (van Bruggen et al., 1996). We now show that binding of nucleosomes to GEnC is in part mediated by HS on the cell surface. We did not detect an additional effect of antibodies on the binding of nucleosomes to GEnC which is compatible with the fact the GEnC line we used does not express Fc gamma receptors. It is interesting to note that the binding of nucleosomes

to GEnC was able to induce complement activation on the GEnC. This activation occurs via the classical pathway of complement. This finding is in agreement with the observation that nucleosomes bound to GEnC dramatically increased the binding of C1q, which induced C3 activation.

Based on this set of studies one could reason that the direct binding of C1q to GEnC *in vivo* is not expected to result in any significant complement-mediated renal injury. On the other hand one can reason that deposition of large amounts of nucleosomes to the kidney potentially could be harmful. We then asked the question whether autoantibodies against C1q and/or nucleosomes could enhance the degree of complement activation on C1q and or nucleosomes bearing GEnC. We used heat inactivated serum from patients with SLE as a source of anti-C1q and anti-DNA autoantibodies. In general all sera of patients with SLE have significant levels of anti-DNA antibodies and only a certain percentage of the sera also have anti-C1q autoantibodies in addition to anti-DNA autoantibodies. However, we were not able to find sera from SLE patients with anti-C1q without anti-DNA. Therefore, we could only assess the combined effect of anti-C1q and anti-DNA autoantibodies on cells sensitized with C1q, nucleosomes or a combination. The results depicted in Fig. 6 indicate that anti-C1q autoantibodies can support activation of complement to a modest extent on GEnC that are sensitized with C1q. However, the effect of sensitization with nucleosomes is superior. The results further show that the effect of anti-DNA and anti-C1q are additive in activation of complement by the classical pathway.

Taken together, our data suggest a more vigorous injurious effect of anti-nucleosomes in combination with anti-C1q antibodies on glomerular injury, which could explain why patients with anti-C1q autoantibodies have a high association with renal disease (Siegert et al., 1992; Trendelenburg et al., 2006). Studies by Trouw et al. (2004a) have shown that C1q is present in glomeruli of normal mice and absent in C1q^{-/-} or RAG^{-/-} mice. Raising the level of IgG in the circulation of RAG^{-/-} mice by splenic cell transfer or infusion of normal IgG resulted in the presence of C1q in the glomerulus of these mice. This would indicate that under normal conditions C1q is present in the glomerulus presumably caused by the presence of trace amounts of IgG in the glomerulus. Whether some part of the C1q in the glomerulus is bound to the endothelial cell, however, is not fully clear at the moment. Of course these *in vitro* findings cannot be extrapolated directly to the *in vivo* situation and, therefore, further analysis of the combined effects of anti-C1q and anti-DNA in *in vivo* models of renal injury are required.

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