Progression of lupus-like disease drives the appearance of complement-activating IgG antibodies in MRL/lpr mice

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Abstract

Objectives. Nucleic acids are known to induce complement activation, which results in the masking and removal of apoptotic cells exposing nuclear components. Dysregulation of these events is characteristic of SLE, a systemic autoimmune disease characterized by the appearance of ANAs. In this study, we aimed to investigate the relationship between development of ANAs and their effect on complement activation by nucleic acids.

Methods. We used protein array technology to characterize complement activation by murine mAbs and polyclonal antibodies against various forms of nucleic acid. Serum samples from MRL/lpr mice were collected, starting before the onset of the disease till 6 months of age. Binding of IgG and its subclasses to dsDNA, ssDNA, RNA, plasmid DNA and nucleosome complexes was determined, along with C3 fixation.

Results. We show that complement C3 binding to various forms of nucleic acid that serve as targets in lupus is absent in normal serum. The addition of dsDNA-specific mAbs to normal serum results in the deposition of complement C3 to nucleic acids. In MRL/lpr mice, IgG antibodies against various nuclear antigens appear with ageing and disease progression. C3 binding to the antigens is somewhat delayed and suggests that accumulation or maturation of pathogenic antibodies is required for inducing C3 binding to ICs containing nucleic acids.

Conclusions. C3 deposition on nuclear antigens, therefore, reflects the state of disease progression in this murine model of SLE.

Key words: Anti-DNA antibody, Autoantibody, Complement activation, Complement C3 component, Lupus.

Introduction

Cellular death by apoptosis or necrosis continuously exposes intracellular components to be recognized by the immune system. An arsenal of molecules contributes to the tagging of dying cells and thereby directs immune responses appropriately: apoptotic cells are cleared off silently, while necrotic cells trigger inflammatory events [1]. Complement is heavily involved in the recognition, tagging and clearance of dead cells with both activatory and inhibitory molecules taking part: serum amyloid protein [2], mannose-binding lectin [3], C1q [4, 5], L- and H-ficolin [6], CRP [7] and properdin [8] are positive regulators of the complement cascade while C4-binding protein and factor H [9] exert negative effects. Composition of the complement deposits on cells in the early apoptotic, late apoptotic and secondary necrotic stages has been shown to differ: C1q binds weakly to early apoptotic cells, its amount increasing on late apoptotic cells when other components such as mannosel-binding lectin, C4 and C3 are also observed [10–12]. Importantly, initiation of classical pathway activation by apoptotic cells does not lead to the generation of the...
membrane attack complex of the terminal pathway [13]. On the contrary, complement binding is an early feature of necrotic cells [14].

Defective phagocytosis of apoptotic cells and debris coupled with lack of generation of tolerogenic signals can result in the generation of pathological immunity to self-components [15], as exemplified by the disease SLE. A hallmark of SLE is the generation of IgG antibodies against dsDNA and nucleosomes. Recent evidence suggests that these antibodies are indeed the result of antibody maturation by somatic hypermutations selected for DNA binding [16, 17]. Anti-DNA antibodies can deposit in the glomerular basal membrane via interaction with DNA itself or with non-DNA glomerular antigens, initiating kidney damage by inducing inflammation [18].

Thus, quality of DNA-directed antibodies and the nature of complement activation associated with cell death can delineate physiology and pathology. The capability of anti-DNA IgG to fix complement has been associated with disease activity [19, 20], LN [21, 22] or, on the contrary, found not to be directly related to disease severity [23, 24]. We have recently reported an array-based technology for the parallel measurement of antibody binding and complement activation by specific antigens [25]. Utilizing arrays allowed us to reliably compare different forms of nucleic acid antigen with respect to antibody binding and complement activation. Here we show, using antigen microarray technology and a murine SLE model, that complement C3 deposition on nucleic acids is profoundly changed when dsDNA-specific IgG autoantibodies are present in the serum.

Materials and methods

Antibodies and reagents

Mouse anti-dsDNA monoclonal IgG2a antibody (MAB030) was obtained from Millipore (Billerica, MA, USA); 10F10, an IgG1 monoclonal, was produced in our laboratory [26]. For experiments with mouse serum Alexa 647-conjugated goat anti-mouse IgG (H+L) (Invitrogen, Carlsbad, CA, USA) and FITC-conjugated F(ab’2) fragment of goat anti-mouse C3 (MP Biomedicals, Irvine, CA, USA) antibodies were used. dsDNA was purchased from Sigma (St. Louis, MO, USA) as calf thymus DNA. Denatured ssDNA was prepared from dsDNA by incubating it at 95°C for 10 min followed by its immediate cooling on ice. RNA was isolated from Ficoll-purified human peripheral blood lymphocytes of a healthy blood donor, using a commercially available kit from Qiagen (Alameda, CA, USA), following the manufacturer’s instructions. Purified plasmid DNA pGEM-5Zf(+) was obtained from Promega (Madison, WI, USA). Nucleosome extracts were generated as described [27] from the human lymphoma BL41; solutions used contained 0.6 mg/ml DNA based on OD260 value. Isotype-specific secondary antibodies were obtained from Southern Biotech (Birmingham, AL, USA) (goat anti-mouse IgG2b-biotin, goat anti-mouse IgG3-biotin, goat anti-mouse IgG2a-Cy5) and Invitrogen ( goat anti-mouse IgG1-Alexa 488), PE-Cy5.5-conjugated streptavidin was purchased from eBioscience (San Diego, CA, USA).

Mice and serum samples

For the experiments with murine serum, we sacrificed C57BL/6, BALB/c (Charles Rivers Laboratory, Wilmington, MA, USA) mice or collected serum from MRL/lpr mice (The Jackson Laboratory, Bar Harbor, ME, USA) of the indicated ages by bleeding from the orbital venous plexus in light anaesthesia. Three MRL/lpr mice had to be euthanized before the end of the study because of the severity of disease. Mice were maintained under specific pathogen-free conditions. All procedures were carried out in accordance with national and institutional regulations. All serum samples were stored at −70°C until use. Proteinuria was measured every 2 weeks using Combi-screen strips (Analytic Systems Biotechnologies, Lichenfels, Germany) and graded semi-quantitatively (0: none; 1: 30–100; 2: 100–300; 3: 300–500; and 4: >500 mg/l).

Experiments were conducted with full compliance with local and national regulations; permissions from the Department of Animal Welfare of the Hungarian Animal Health and Animal Welfare Directorate and from the Bulgarian National Commission for Ethical Work with Laboratory Animals were obtained.

Microarray production and measurements

Either homemade nitrocellulose-covered glass slides or 16-pad FAST slides (GE Healthcare, Piscataway, NJ, USA) were used for the assays. The following materials were printed on the slides with the indicated starting concentrations: calf thymus DNA (Sigma) at 1 mg/ml, in native (dsDNA) and denatured form (ssDNA); RNA isolated from human peripheral blood lymphocytes, 0.7 mg/ml; plasmid DNA, 0.2 mg/ml; nucleosome extract, 0.6 mg/ml DNA content; goat anti-mouse C3 (MP Biomedicals, Irvine, CA, USA) and goat anti-mouse IgG at 1 mg/ml. Features were printed in triplicate of 1/5 serial dilutions by Bio-Odyssey Calligrapher miniarrayer (Bio-Rad, Hungary) using solid pin and cooled platform, then stored at 4°C in sealed bags. Microarray experimental procedures for murine serum samples have been published [28], and we used this protocol with little modifications. For serum treatment, MAB030 anti-DNA mAb solution was added at 2% to normal mouse serum, and 10F10 was used at a final concentration of 10 μg/ml. Fluorescently labelled anti-mouse C3 antibody was diluted 10 000-fold, while anti-mouse IgG-detecting antibody 5000-fold, in PBS containing 5% BSA.

Sera were diluted five times in Veronal buffer [727 mM NaCl, 9.12 mM Na-diethyl-barbiturate (C8H11O3N2Na), 15.63 mM 5,5-diethyl barbituric acid (C8H12N2O3), pH 7.3] containing 5% BSA, 0.05% Tween-20 and divalent cations (2.5 mM Ca2+, 0.7 mM Mg2+). Serum-treated slides were washed in PBS with 0.05% Tween-20, then incubated in the mixture of anti-mouse C3-FITC and anti-mouse IgG-Alexa 647 detection antibodies. Labelling with fluorescent antibodies was carried out at room temperature for 30 min in PBS containing 5% BSA.
and 0.05% Tween-20. Following washing in PBS with 0.05% Tween-20, arrays were dried and scanned on Typhoon Trio imager (GE Healthcare) or Axon GenePix 4300A.

Microarray and statistical analysis
Data were analysed with ImageQuant TL (GE Healthcare) or GenePix Pro7 (Molecular Devices, Sunnyvale, CA, USA) software. Signal intensities were calculated by subtracting background from medians of three parallel signal intensities in a spreadsheet program (Microsoft Excel; Microsoft, Redmond, WA, USA).

Statistica software (Statsoft, Tulsa, OK, USA) was used for statistical analysis of the results. The non-parametric Mann–Whitney U-test was applied to the data, and null hypothesis was rejected when $P < 0.05$.

Results
C3 deposition is not detectable on solid-phase adsorbed dsDNA treated with normal serum
Normal serum contains a number of innate molecules that have been shown to bind DNA and initiate complement activation. To our surprise, when we treated antigen arrays containing various nucleic acid antigens with serum, no C3 was detected on most forms of nucleic acid. In fact, C3 signal intensity of dsDNA spots was less than the background C3 signals. This observation was true for both the examined strains of mice (BALB/c, C57BL/6; Fig. 1 and data not shown). Minimal amounts of C3 were detectable on nucleosome preparations only.

Binding of IgG to DNA promotes the deposition of C3
SLE is characterized by the appearance of high-affinity IgG specific for nuclear antigens including dsDNA and the primary or secondary decrease of the concentration of early complement components. To establish the effects of ANAs on our measurements, we examined normal serum supplemented with mAbs specific for dsDNA. As shown in Fig. 1, these IgG-type antibodies reacted with all five tested nucleic acid-containing antigens. Importantly, binding of IgG was accompanied by significant C3 deposition (Fig. 1) in spite of normal complement function in the tested serum.

DNA-bound IgG fixes C3 in an epitope density-dependent manner
Both murine mAbs efficiently induced C3 deposition in normal serum, indicating that the presence of a high-affinity anti-DNA antibody in the serum alone is sufficient to override down-modulation of the complement cascade. However, IgG binding to the antigen and the induced C3 deposition followed different patterns on different antigens (Fig. 1). Thus, epitope density and antibody qualities—such as isotype or affinity—together exert a fine control on complement activation and C3 deposition on DNA-containing ICs.

Appearance of nucleic acid-specific antibodies with disease progression follows a pattern
Serum was collected from lupus-prone MRL/lpr mice at 4-week intervals, starting at 5 weeks of age. Disease progression was confirmed by measuring proteinuria, serum albumin and IgG levels (Table 1). Antibodies against most nuclear antigens started to appear already at 9 weeks of age and their titre continued to increase almost throughout the study. This increase was most prominent for ssDNA and RNA at 9 weeks of age; dsDNA, plasmid DNA and anti-nucleosome-reactive antibodies developed in comparable amounts only at 17 weeks of age (Fig. 2A). In spite of the appearance of DNA-specific antibodies, C3 deposition was only observed with a delay of ~4 weeks (Fig. 2B). This is well reflected by the scatter plot depicting C3 deposition as a function of IgG binding (Fig. 2C): C3 deposition appears when a certain threshold value of IgG binding is exceeded.

dsDNA-specific IgG1 shows the most dramatic increase in time
Reactivity of different subclasses of IgG with nucleic acids was determined using the same arrays as previously. In the mouse, IgG2a is regarded as the isotype with the most potent effector functions mediated via Fc and complement receptors. Interestingly, after an initial increase during the second month of life, dsDNA reactivity of IgG2a did not rise any further (Fig. 3); IgG2b and IgG3 behaved similarly. On the contrary, IgG1 levels against all tested antigen species continued to rise until Week 21, the effect being most pronounced for dsDNA, plasmid DNA and nucleosome reactivity.

Discussion
We have used an array-based approach for assessing complement activation in the presence of natural and pathological antibodies to various forms of DNA. This approach allowed us to compare complement-activating properties of these antibodies and also to simultaneously measure complement C3 deposition induced by the bound antibodies. Of the tested antigens, purified dsDNA has long been used for the identification of pathological autoantibodies associated with SLE; in addition to eukaryotic DNA, we also used bacterially derived plasmid DNA as an alternative form of testing.

C1q plays a significant role in the normal physiological clearance of apoptotic cells in vivo [29] and was shown to bind DNA [30]. We used three forms of eukaryotic DNA that are slightly different in structure and composition: dsDNA, denatured ssDNA and nucleosome complexes. Notwithstanding earlier observations that contact of DNA with serum triggers complement activation, C3 fragments were either not detectable or present in modest amounts (Fig. 1). This can be interpreted as a lack of C3 convertase formation or as the inability of C3b to covalently bind to acceptor sites. Negative regulators capable of inhibiting C3 convertase generation have been shown...
Fig. 1 Deposition of complement C3 fragments to various forms of nucleic acids. RNA, ssDNA, dsDNA, plasmid DNA and nucleosome were printed on nitrocellulose arrays, which were incubated in normal murine serum alone or supplemented with the indicated anti-dsDNA antibodies. Deposited C3 fragments were visualized by fluorescently labelled antibodies. Images are false colour overlays and are representative of more than three independent measurements. Diagrams display median relative fluorescence units (RFU) of triplicates in one representative experiment; (—): connect IgG; (- - -): connect C3 values.
**FIG. 2** Appearance of serum nucleic acid reactivity during the development of SLE-like disease. IgG binding (A) and C3 deposition (B) to the indicated nucleic acid antigens was determined from sera collected over a period of 20 weeks. (C): individual animals; (*): significant difference from the first time point. Two-dimensional display of IC composition (C) shows the dependence of complement activation on IgG binding. RFU: relative fluorescence units.

**TABLE 1** Markers of disease progression

<table>
<thead>
<tr>
<th>Marker</th>
<th>Age of MRL/lpr mice, week</th>
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<tbody>
<tr>
<td></td>
<td>5 (n = 10)</td>
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<tr>
<td>Proteinuria, mg/dl</td>
<td>15 (0–30)</td>
</tr>
<tr>
<td>Serum albumin (normal percentage)</td>
<td>84 (61–139)</td>
</tr>
<tr>
<td>Serum IgG (normal percentage)</td>
<td>115 (59–193)</td>
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To confirm the development of SLE-like disease in MRL/lpr mice, proteinuria, serum albumin levels and serum IgG levels were monitored. Albumin and IgG levels are expressed as percentage of reference values obtained from 25-week-old BALB/c mice. *Significant change compared with initial values (5 weeks) at \( P < 0.05 \) (Mann–Whitney U-test); medians and ranges are shown.
to bind to DNA [31] and are responsible for the down-regulation of complement.

The role of C3 in IC diseases is complex and somewhat controversial. Mice genetically deficient in C3 on an MRL/lpr background showed increased proteinuria and IgG deposition in the glomeruli [32]. Similarly, in a kidney transplant model using sheep anti-glomerular basement membrane antibodies to induce IC deposition in the kidneys, absence of C3 in the circulation was associated with higher amounts of glomerular ICs and more severe disease [33]. These studies point to the beneficial role that C3 may play via enhancing IC clearance. On the other hand, in mice on an IgG Fc receptor IIB-deficient B6 background, the absence of C3 strongly inhibited anti-DNA production [34], suggesting that C3 deposition on nuclear autoantigens plays an important role in the amplification of anti-DNA autoantibody production, at least in the absence of IgG-mediated negative feedback. In the light of our observations, exposure of DNA on apoptotic cells to body fluids plays little, if any, role in the deposition of C3 under physiological conditions.

The presence of monoclonal or polyclonal dsDNA-specific IgG in the serum was associated with increased C3 deposition on antigens containing DNA (Figs 1 and 2). The anti-dsDNA mAbs were tested (Fig. 1) and an additional panel of seven such antibodies also reacted with ssDNA and nucleosome [35]; the latter were also shown to react with nuclear fragments and apoptotic blebs. These data suggest that epitopes recognized by these dsDNA-specific antibodies are accessible in denatured and histone-bound DNA, exposed on apoptotic and necrotic cells. This reactivity is thought to contribute to the pathogenesis of LN [36, 37]. Emerging evidence suggests that these anti-dsDNA antibodies are not merely polyreactive,
but are generated during a normal immune response, as a result of somatic hypermutation-mediated affinity maturation [38, 39]. Nucleosomes that have been cleaved and modified during the process of apoptosis, escape normal clearance in SLE and encompass epitopes that normally are not encountered by the immune system, and then might subsequently positively select high-affinity anti-DNA B cells.

In spite of the early appearance of DNA-specific antibodies, complement activation becomes detectable with a delay of 2–4 weeks (Fig. 2). It is more likely that not qualitative but quantitative changes (Figs 2 and 3) result in the presence of C3 in the ICs. Pathogenic dsDNA antibodies are per definitionem high-affinity binders. They are expected to be able to displace DNA-recognition machinery of the innate immune system by competition and thereby alter complement-activating properties of DNA. Thus, the presence of DNA-specific, high-affinity IgG can shift the balance from non-inflammatory, phagocytic signals to the generation of pro-inflammatory anaphylatoxins and membrane attack complexes along with the provision of ligands for activatory Fc receptors. In addition to displacing complement regulators, DNA-bound IgG can enhance C3 deposition by serving as an acceptor molecule for C3b. The dimeric C3b captured by IgG is a C5 convertase pre-cursor [40], so DNA-bound C3b-IgG could promote progression of complement activation beyond C5 cleavage. The ability of anti-DNA IgG to induce C3 deposition in our assay may therefore serve as an indicator of disease activity or disease progression.

In summary, our data indicate that the presence of dsDNA-specific IgG antibodies dramatically changes complement activation by DNA. Complement C3 can contribute to both the generation of these antibodies and their pathogenicity. Importantly, this phenomenon could be exploited for the fine characterization of autoantibodies in humans using antigen microarrays [41].

### References