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Autoantibodies against Complement C1q Specifically Target C1q Bound on Early Apoptotic Cells

Cornelia Bigler, Monica Schaller, Iryna Perahud, Michael Osthoff, and Marten Trendelenburg

Autoantibodies against complement C1q (anti-C1q) are frequently found in patients with systemic lupus erythematosus (SLE). They strongly correlate with the occurrence of severe lupus nephritis, suggesting a pathogenic role in SLE. Because anti-C1q are known to recognize a neoepitope on bound C1q, but not on fluid-phase C1q, the aim of this study was to clarify the origin of anti-C1q by determining the mechanism that renders C1q antigenic. We investigated anti-C1q from serum and purified total IgG of patients with SLE and hypocomplementemic urticarial vasculitis as well as two monoclonal human anti-C1q Fab from a SLE patient generated by phage display. Binding characteristics, such as their ability to recognize C1q bound on different classes of Igs, on immune complexes, and on cells undergoing apoptosis, were analyzed. Interestingly, anti-C1q did not bind to C1q bound on Igs or immune complexes. Neither did we observe specific binding of anti-C1q to C1q bound on late apoptotic/necrotic cells when compared with binding in the absence of C1q. However, as shown by FACS analysis and confocal microscopy, anti-C1q specifically targeted C1q bound on early apoptotic cells. Anti-C1q were found to specifically target C1q bound on cells undergoing apoptosis.

Our observations suggest that early apoptotic cells are a major target of the autoimmune response in SLE and provide a direct link between human SLE, apoptosis, and C1q. The Journal of Immunology, 2009, 183: 0000–0000.

S
ystemic lupus erythematosus (SLE) is a systemic autoimmune disease characterized immunologically by a variety of autoantibodies, B cell hyperactivity, and immune complex (IC) formation (1, 2). Complement, especially C1q, the first component of the classical pathway of complement, is considered to be involved in the pathogenesis of SLE. This view is based on the following observations: First, almost all patients with C1q deficiency develop a lupus-like syndrome, with homozygous C1q deficiency being the strongest disease susceptibility gene for the development of SLE (3, 4). Second, a substantial number of patients with SLE develop hypocomplementemia with depletion of C1q and other components of the classical pathway of complement (5) and C1q is deposited in affected tissues (6, 7). Last, in about one-third of unselected SLE patients, autoantibodies to C1q can be detected, and they are strongly associated with the occurrence of severe lupus nephritis as well as the consumption of C1q (8–10).

A possible explanation for the strong link between complement C1q and SLE is the so-called “waste disposal” hypothesis (11, 12). This hypothesis assumes that SLE is driven by a defective clearance of dead and dying cells that could become antigenic and drive autoimmunity (13, 14). Experimental support for this hypothesis stems from the fact that mice with defined single gene defect leading to an altered clearance of apoptotic cells develop severe autoimmunity with the occurrence of autoantibodies directed against nuclear components as seen in SLE patients (15). Vice versa, lupus-prone mice were shown to have an intrinsic impairment in apoptotic cell uptake (16), and macrophages derived from the peripheral blood of SLE patients also had a defective uptake of apoptotic cells (17). In addition, a number of lupus autoantigens could be located on the surface of apoptotic bodies and apoptotic blebs (18), and the injection of an excess of apoptotic cells in healthy mice could induce the production of autoantibodies (19) such as anti-nuclear, anti-ssDNA, and anti-cardiolipin Abs. Therefore, apoptotic cell debris seems to be the source of autoantigens in SLE. Independently, C1q has been described to be involved in the clearance of self-Ags generated during apoptosis by binding specifically to apoptotic keratinocytes and vascular endothelial cells (20–25). Furthermore, an accumulation of apoptotic cells could be observed in kidneys from C1q-deficient mice. These findings suggested that in addition to its role in the clearance of IC, complement C1q is also involved in the prevention of autoimmunity through a role in the disposal of dead and dying cells.

However, although there are strong links from C1q to SLE, from SLE to apoptosis, and from apoptosis back to C1q, no direct link of all three (SLE, C1q, and apoptosis) has been established. Furthermore, because most patients with SLE do not have a primary deficiency of complement C1q, the pathogenic link between C1q and human SLE is not well understood.

Low levels of C1q, as often observed in human SLE, are due to the consumption of early components of the classical pathway of complement (5). Secondary hypocomplementemia in SLE is most often associated with autoantibodies against C1q (9, 10, 26, 27). Anti-C1q have been shown to strongly correlate with the occurrence of biopsy-proven active lupus nephritis (9), and severe forms of lupus nephritis in the absence of anti-C1q are rare (28). These...
findings suggest that anti-C1q have an active role in the pathogenesis of SLE. Anti-C1q Abs were first recognized in 1971 (29) and identified in 1988 (30, 31). They were mostly seen in patients with SLE, but the highest titers have been observed in patients with the hypocomplementemic urticaria vasculitis syndrome (HUVS) that is closely related to human SLE (8, 32). In a comparative study, no apparent differences between the binding characteristics of anti-C1q from patients with SLE and HUVS could be found (33). The Abs were mostly of the IgG isotype (34, 35) and, in contrast to the binding of IC, bound to the collagen-like region of C1q. The binding was of high affinity and mediated by Fab. As shown for most of the other lupus autoantibodies, no cross-reactivity of anti-C1q with other Ags could be identified (36, 37). In addition, our recent analysis of human anti-C1q mAbs generated by phage display from the bone marrow of a patient with SLE suggests that anti-C1q Abs are specific, of high affinity, and the result of an Ag-driven immune response (M. Schaller, C. Bigler, D. Danner, H. J. Ditzel, and M. Trendelenburg, submitted for publication).

Interestingly, anti-C1q bind to a neoepitope that, due to conformational changes, is only expressed on bound C1q. However, the precise epitope cannot yet be identified, and the mechanism that renders C1q antigenic had not been clarified. Because SLE is an IC disease and because C1q has originally been described to be involved in the clearance of IC, the most likely mechanism is that C1q after having bound to IC expresses one or several neoepitopes that are targeted by anti-C1q. However, in the context of an impaired clearance of apoptotic material, it is also possible that C1q bound to the surface of apoptotic bodies becomes antigenic itself, similar to nuclear components that are normally not exposed to the immune system. Therefore, understanding the conditions under which the neoepitope of C1q is exposed, leading to an anti-C1q autoimmune response in SLE, might provide a better understanding of the pathogenic mechanisms in SLE.

Materials and Methods

Human anti-C1q autoantibodies

**Patient serum.** As a source of anti-C1q, we used serum from patients with either SLE or HUVS. SLE patients fulfilled at least 4 of the 11 criteria of the American College of Rheumatology (38, 39). HUVS patients had been described previously (8). Sera from healthy blood donors were used as a control.

**Purified total IgG.** Total IgG was purified from a healthy blood donor, as well as from one of the above-described patients with HUVS (case 1 in Ref. 8), having developed mesangiproliferative glomerulonephritis, and two SLE patients, both having developed proliferative glomerulonephritis, by protein G affinity chromatography. The HUVS patient had low-level ANA (1:80, speckled) and was negative for Abs against dsDNA, phospholipids, and nucleosomes.

**Preparation of human Fab anti-C1q Abs by phage display.** As described in a separate study, a bone marrow-derived IgG1 κλ (IgGk/IgGλ) Fab phage display library from a SLE patient with high anti-C1q Ab titer had been screened against purified human C1q by ELISA. In short, after five panning rounds, the phagomid DNA from the third and fourth rounds of panning was prepared and the gene III fragment was removed by restriction enzyme digest, followed by transformation of XL1-blue cells to produce soluble IgG1 Fabs (M. Schaller, C. Bigler, D. Danner, H. J. Ditzel, and M. Trendelenburg, submitted for publication).

The screening yielded six Fabs showing the typical binding characteristics described for anti-C1q: the Fabs exhibited strong binding (affinity showing $K_d$ in the range of $8.4 \times 10^{-8}$ M to 1.4 $\times 10^{-5}$ M) to the neoepitopes expressed by polystyrene-bound C1q that could be located on the collagen-like region of the molecule. For the experiments presented in this study, we used the clones A14 and B8 because they showed the highest signals of the six anti-C1q Fabs generated when tested for binding to C1q in ELISA. The purity of the anti-C1q Fabs is demonstrated in supplemental Fig. 1A.3

**Anti-C1q control Abs**

For the detection of deposited C1q, different control Abs were used, as follows.

**Mouse anti-C1q mAbs.** Murine anti-human C1q mAbs were generated by the immunization of C1q-deficient mice backcrossed for at least 10 generations on a BALB/c genetic background (gift of M. Botto, Imperial College, London, U.K.) with purified human C1q (gift of Bühlmann Laboratories, Schönenbuch, Switzerland) >99% pure as judged by SDS-PAGE, demonstrated in supplemental Fig. 1B) in collaboration with T. Rolink (Immunology, Department for Biomedicine, University of Basel, Basel, Switzerland). In short, two groups consisting of three mice each were immunized s.c. with either 20 $\mu$g of C1q alone or 20 $\mu$g of C1q bound on heat-aggregated IgG generated from purified total IgG of healthy BALB/c mice. One mouse in each of the two groups showing the highest levels of anti-C1q in serum as judged by ELISA (see below) was sacrificed, and the spleen was used to generate hybridomas according to a standard protocol (40).

Ab-secreting hybridoma cells were screened for anti-C1q by ELISA. In short, human C1q was coated overnight on ELISA plates (Nunc) at a concentration of 0.5 $\mu$g/ml. Supernatants of clones were diluted 1/1 in PBS/Tween 20 (0.05%), 1% FCS containing 1 M NaCl. After incubation, plates were washed and bound IgG was detected using biotinylated polyclonal goat anti-mouse IgG (Southern Biotechnology Associates) and HRP-launched streptavidin (Jackson ImmunoResearch Laboratories). Before expansion, anti-C1q-producing hybridoma cell lines were selected by limited dilution and repeated testing for anti-C1q production. Nine (Fig. 1) clones producing Abs specific for human C1q could be generated that were able to bind C1q even under high salt conditions, i.e., in the presence of 1 M NaCl. All clones were also positive for murine C1q and showed no cross-reactivity, as judged by immunohistochemical staining of spleen sections

4 The online version of this article contains supplemental material.
from normal BALB/c mice compared with Clq-deficient mice (data not shown). Interestingly, whereas the binding of most of the generated murine anti-human Clq mAbs to plate-bound Clq could be inhibited by the pre-incubation with fluid-phase Clq, 2 of the 14 clones (clones 23D11 and 34A4) could not be inhibited by fluid-phase Clq even in Ag excess (Ab:Ag ratio in the range of 1.5–20). This lack of inhibition by fluid-phase Clq indicated the binding to neoepitopes that are only expressed on bound Clq, but not on fluid-phase Clq. Binding of Abs with the strongest signal to plate-bound Clq with or without preincubation with fluid-phase Clq is demonstrated in Fig. 1. For experiments performed in this study, clones 12F10 and 23D11 were used. Whereas 12F10 bound Clq independent of its deposition, 23D11 only bound to neoepitopes expressed on solid-phase Clq. Clones 12F10 and 23D11 were used to verify the successful binding of Clq to Igs, IC, heat-aggregated IgG (HAGG), polystyrene beads, and phosphatidylserine in ELISA. Clones 12F10 and 23D11 could not be used to verify the successful binding of Clq to dsDNA and nucleosomes due to competition for the same epitopes.

Commercially available anti-Clq Abs. A murine mAb to purified human Clq (Quidel) was used to verify the successful binding of Clq to nucleosomes and dsDNA in ELISA. The same Ab was FITC labeled (FluoroTag FITC conjugation kit; Sigma-Aldrich), according to the supplier’s instructions, and used for the detection of Clq on apoptotic cells together with a FITC-conjugated rabbit anti-human Clq Ab (DakoCytomation).

Binding of anti-Clq autoantibodies to Clq bound on Ig preparations or IC
Polystyrene ELISA plates (Nunc) were coated with either Clq alone or different unspecific mouse mAbs (IgM, IgA, IgG1, IgG2a, IgG2b, and IgG3) at a concentration of 1 µg/well (Southern Biotechnology Associates), or polyclonal goat anti-clot encoded sera (Quidel) or human polyclonal IgM (Sigma-Aldrich) at 0.5 µg/ml. Alternatively, IC were established by either coating with collagen type I (Sigma-Aldrich; concentration 1 mg/ml) or polyclonal goat IgM (Sigma-Aldrich) at 0.5 µg/ml. As controls, IC were used to establish C1q-dependent binding of anti-Clq to apoptotic cells in an ELISA format with polystyrene beads or HAGG as described in detail elsewhere (Hein-Lewicka et al., manuscript submitted).

Depletion experiment with polystyrene beads or HAGG
Depletion of anti-Clq from serum of a SLE and a HUVS patient being highly positive for anti-Clq was performed using Clq bound to HAGG (IgG was isolated from NHS using a protein G column, then incubated for 30 min at 65°C) vs Clq bound to polystyrene beads imitating the binding of Clq to polystyrene ELISA plates (Polyscience). After pre-incubation of the sera with HAGG-Clq or beads-Clq, the complexes were removed either by ultracentrifugation at 192,000 × g for 1 h at 4°C (HAGG-Clq) or by centrifugation at 3,200 × g for 5 min (beads-Clq), and the supernatants were tested for anti-Clq activity using a conventional anti-Clq ELISA, as described previously (41, 42). The experiments were performed in different dilutions, and values of NHS were used as blanks. Data are expressed as maximal depletion (in %) obtained when compared with sera depleted with HAGG or beads alone.

Cell populations
Jurkat (human T cell leukemia) and Molt 4 (human T cell leukemia) cells were cultured in RPMI 1640 with l-glutamine and 26 mM HEPES (Life Technologies products; Invitrogen) with 10% FCS (Life Technologies products; Invitrogen). For the preparation of human peripheral blood T cells, PBMCs from healthy donors were cultured in RPMI 1640 and 10% FCS, and then stimulated with PHA (REMEL; 1 µg/ml) and IL-2 (Novartis Pharmaceuticals; 500 U/ml). T cells were isolated by CD3-positive selection with MACS beads (Miltenyi Biotec).

Binding of anti-Clq autoantibodies to Clq bound on apoptotic cells
Two million cells/ml were washed twice with RPMI 1640 (Life Technologies products; Invitrogen) without FCS to remove Clq from culture medium. Cells were then seeded in 6-well tissue culture plates (Falcon; BD Biosciences) at a concentration of 5 × 10⁵/cm² before being placed on microscopy slides and irradiated with UV light at 254 nm for 3 min (stratalinker 1800; Stratagene) after incubation for 2–3 h at 37°C and 5% CO₂.

Alternatively, cells were incubated with staurosporine (BioSource International; Invitrogen) at 0.43 µM for 6 h at 37°C and 5% CO₂. After being rendered apoptotic, cells were incubated with 3 µg of purified Clq (Complement Technologies) per 3 × 10⁵ cells and 100 µl/ml in PBS at 37°C for 20 min in 96-well U-bottom tissue culture plates (Falcon; BD Biosciences). Plates were washed with PBS and incubated with the anti-Clq Fab autoantibodies A14 and B8 (as described above) at 2 µg/ml, or with purified IgG from sera of an anti-Clq-positive HUVS patient, or with purified IgF from sera of two anti-Clq-positive SLE patients or a healthy blood donor at 500 µg/ml in PBS for 20 min at 37°C. Anti-Clq-dependent binding of anti-Clq to apoptotic cells was detected by staining with an anti-human PE-conjugated F(ab')₂ specific for F(ab')₂ (Jackson Immunoresearch Laboratories) or goat anti-human IgG specific for the Fcγ fragment PE-conjugated F(ab')₂ (Jackson Immunoresearch Laboratories) in PBS for 15 min at room temperature in the dark. Cells were then washed with PBS and stained with FITC-conjugated rabbit anti-human Clq (DakoCytomation) or with a FITC-labeled murine mAb to human Clq (Quidel) for 15 min at room temperature in the dark. Finally, to identify apoptotic cells, cells were stained with annexin V or propidium iodide (BD Pharmingen).

Confocal microscopy
Cells were stained, as described above, except that the anti-Clq Fab autoantibody A14 was used at a concentration of 3 µg/ml. The cells were fixed in 2% paraformaldehyde (BioSource) before being placed on microscopy slides (Erie Scientific/Thermo Fisher Scientific) and dried. VECTASHIELD mounting medium with 4'-6-diamidino-2-phenylindole (Vector Laboratories) was added, and cells were visualized by confocal microscopy with a pinhole of 1.5 µm.

Anti-Clq autoantibody binding to Ags expressed on apoptotic cells
Binding of the two human anti-Clq Fab autoantibodies (1 µg/ml) to Clq bound to human placental dsDNA (Sigma-Aldrich) and phosphatidylserine (Fluka) was assessed. Furthermore, binding of anti-Clq from patient sera to Clq bound to dsDNA was tested. DNA and phosphatidylserine had been coated onto Costar microriter plates in 1% BSA-PBS at a concentration of 2 µg/well (DNA) or 5 µg/ml in 100% methanol (phosphatidylserine) in a total volume of 100 µl overnight at 37°C (DNA) or at room temperature (phosphatidylserine), respectively. The plates were then washed with PBS-0.05% Tween 20 and blocked with 3% BSA in PBS for 1 h at 37°C before incubation with purified human Clq at 0.5 µg/ml in PBS for 2 h at 37°C. The plates were then washed again before adding anti-Clq Fab autoantibodies (1 µg/ml) or sera from an anti-Clq-positive HUVS patient or a healthy donor (1/50 in high salt buffer). In parallel, deposited Clq was detected by a monoclonal mouse anti-Clq (clone 23D11 or Quidel) diluted 1/1000 in PBS to verify the successful binding of Clq. The detection of the Abs was conducted, as described above.

Furthermore, we analyzed whether nucleosomes could be the binding partner of Clq, allowing exposure of the neoepitope relevant for the binding of anti-Clq Abs by using a modified commercially available ELISA kit.
(ORGENTEC Diagnostika). As described above, C1q was allowed to bind to the plate precoated with nucleosomes isolated from human cell cultures. The binding of anti-C1q Fab autoantibodies or anti-C1q from sera of a HUVS patient or a healthy donor was then analyzed. The detection of the Abs and the calculation were conducted, as described above.

Statistical analysis
To determine statistical differences between the C1q-dependent binding of anti-C1q preparations to the different apoptotic cell populations, two-tailed Mann-Whitney tests were performed. For multiple comparisons, we used a one-way ANOVA Bonferroni test. All tests were performed using GraphPad Prism version 4 (GraphPad).

Results
Anti-C1q autoantibodies do not recognize C1q bound to Igs or IC
Assuming that neoepitopes of C1q targeted by anti-C1q would be expressed once C1q has bound to Igs/IC, in vitro constructs of Igs
and IC, respectively, were tested for binding of anti-C1q in the presence or absence of C1q Ag. As shown in Fig. 2A, all patients had high titers of anti-C1q. Anti-C1q Abs recognize C1q neither on monoclonal nor on polyclonal Ig preparations (Fig. 2B and C) nor on different IC (Fig. 2D and E), despite abundant C1q deposition as verified by the binding of mouse monoclonal anti-C1q (23D11 and 12F10). The lack of binding was not due to a lack of expression of neoepitopes because the mouse monoclonal anti-C1q 23D11 specifically recognizes a neoepitope expressed on bound C1q (see Materials and Methods). The Iggs/IC were able to activate complement as verified by C4d deposition, suggesting a C1q deposition resembling the physiological binding to IC. However, C4d deposition cannot explain the lack of binding of anti-C1q in our assays because high salt conditions as used for the incubation steps completely prevented activation of the classical pathway of complement (data not shown).

To confirm the results described above, we set up depletion experiments with polystyrene beads and heat-aggregated IgG (HAGG) that served as a model for IC. Anti-C1q-positive sera could substantially be depleted by C1q bound to polystyrene beads, imitating the situation of C1q bound to polystyrene ELISA plates. However, anti-C1q could not be depleted by C1q bound to HAGG, although both C1q on polystyrene beads and C1q on HAGG were able to deplete mouse anti-C1q 12F10 and 23D11 mAbs (Fig. 3).

Additionally, no C1q-dependent binding of anti-C1q to C1q when bound on laminin, heparan sulfate, fibronectin, peptide 2F, and collagen I and IV could be observed, indicating that neither Iggs/IC nor those proteins are able to present C1q such that it can be recognized by anti-C1q autoantibodies (data not shown).

Human anti-C1q Fab autoantibodies recognize C1q on early apoptotic cells

Having shown that C1q bound on Iggs/IC is not targeted by anti-C1q, C1q bound on apoptotic cells was investigated as a potential target for anti-C1q.

As described by others (21, 43), C1q mostly bound to late apoptotic/necrotic cells (double positive for annexin V and 7-AAD), and IC, respectively, were tested for binding of anti-C1q in the presence or absence of C1q Ag. As shown in Fig. 2A, all patients had high titers of anti-C1q. Anti-C1q Abs recognize C1q neither on monoclonal nor on polyclonal Ig preparations (Fig. 2B and C) nor on different IC (Fig. 2D and E), despite abundant C1q deposition as verified by the binding of mouse monoclonal anti-C1q (23D11 and 12F10). The lack of binding was not due to a lack of expression of neoepitopes because the mouse monoclonal anti-C1q 23D11 specifically recognizes a neoepitope expressed on bound C1q (see Materials and Methods). The Iggs/IC were able to activate complement as verified by C4d deposition, suggesting a C1q deposition resembling the physiological binding to IC. However, C4d deposition cannot explain the lack of binding of anti-C1q in our assays because high salt conditions as used for the incubation steps completely prevented activation of the classical pathway of complement (data not shown).

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As described by others (21, 43), C1q mostly bound to late apoptotic/necrotic cells (double positive for annexin V and 7-AAD),
whereas there were only few C1q-positive early apoptotic cells (annexin V single positive) after preincubation with purified human C1q (Fig. 4D).

As shown in Fig. 5, in a population of Jurkat cells that had been rendered apoptotic by UV light irradiation, all cells being still viable (double negative for annexin V and 7-AAD) were negative for C1q and anti-C1q Fab binding. In contrast, we observed binding of anti-C1q Fab autoantibodies A14 and B8 to C1q on early apoptotic (annexin V-positive, 7-AAD-negative) cells when compared with binding in the absence of C1q. In the presence of C1q, a median of 66.41% cells bearing C1q was also positive for anti-C1q Fab autoantibody A14 (range: 40.42–78.70), whereas a median of 76.0% cells bearing C1q was positive for anti-C1q Fab autoantibody B8 (range: 53.40–91.80). On late apoptotic/necrotic cells, we also observed cells being positive for both C1q and anti-C1q Fab autoantibodies. However, in contrast to early apoptotic cells, late apoptotic/necrotic cells also revealed binding in the absence of C1q Ag, suggesting additional unspecific binding (Fig. 5). To quantify the C1q-dependent binding of anti-C1q, we calculated the relative increase of binding of A14 and B8 to apoptotic cells in the absence vs binding in the presence of C1q. Whereas A14 and B8 showed clear C1q-specific binding to early apoptotic cells, we could not demonstrate C1q-dependent binding to late apoptotic/necrotic cells (Fig. 6). These results could be confirmed using Jurkat cells being rendered apoptotic with staurosporine (Fig. 7A) and by using another T cell line (Molt 4 cells) as well as using peripheral blood T cells as a source for apoptotic cells (Fig. 7, B and C).

Purified total IgG from an anti-C1q-positive HUVS patient as well as from two SLE patients also showed C1q-specific binding to early apoptotic cells.

To confirm the results obtained with human anti-C1q Fab autoantibodies, we also tested purified IgG from a HUVS patient with high titers of anti-C1q Abs, but undetectable anti-dsDNA, anti-phospholipid, and anti-nucleosome Abs compared with IgG from a healthy donor. IgG from the HUVS patient showed significant C1q-dependent binding to early apoptotic cells, but again there was no quantifiable C1q-dependent binding to late apoptotic/necrotic cells, confirming our results seen with anti-C1q Fab autoantibodies. Purified IgG from two anti-C1q-positive SLE patients showed similar results, although more binding of IgG to early and especially to late apoptotic/necrotic cells in the absence of C1q was observed. In contrast, IgG from a healthy
donor showed no C1q-dependent binding either to early apoptotic or to late apoptotic/necrotic cells (Fig. 8 and supplemental Figs. 2 and 3).

C1q and anti-C1q Fab autoantibodies colocalize on intact apoptotic cells

To analyze whether C1q and anti-C1q Fab autoantibodies indeed colocalize on apoptotic cells, confocal microscopy was performed. As expected, abundant C1q deposition to dying cells could be observed (Fig. 9B). The staining patterns varied between a more patchy staining for C1q on cell debris and more circular staining on apparently intact cells. Interestingly, colocalization of C1q and anti-C1q Fab autoantibodies was primarily found on apoptotic cells with more circular C1q staining that had an intact cell membrane and an intact nucleus most likely representing cells in early stages of apoptosis (Fig. 9D). As control, viable cells stained negative for C1q and anti-C1q Fab autoantibodies (Fig. 9, B and C).

Human Fab anti-C1q autoantibodies do not recognize C1q on dsDNA, phosphatidylserine, or nucleosomes

To elucidate potential receptors of C1q on apoptotic cells that might be responsible for the expression of neoepitopes relevant for the binding of anti-C1q autoantibodies, we tested the binding of anti-C1q Fab autoantibodies and anti-C1q-positive serum from a HUVS patient (undetectable anti-dsDNA, anti-phospholipid, and anti-nucleosome Abs) to C1q bound on phosphatidylserine, dsDNA, and nucleosomes. A control monoclonal mouse anti-C1q clone 23D11 and polyclonal mouse anti-C1q were tested in the same ELISA, confirming that C1q was indeed able to bind to those autoantigens. As demonstrated in Fig. 10, anti-C1q Fab autoantibodies did not recognize C1q bound to phosphatidylserine, nor bound to dsDNA, nor bound to nucleosomes, indicating that none of those autoantigens tested is able to present C1q in an anti-C1q suitable conformation. Similarly, no C1q-dependent binding of sera from an anti-C1q-positive HUVS patient to nucleosomes and dsDNA could be observed (data not shown).

Discussion

Previous studies suggested a link from SLE to apoptosis, from apoptosis to complement C1q, and from C1q back to SLE. These links are based on observations that apoptotic cells are a source of autoantigens and that SLE indeed is associated with a defective clearance of dead and dying cells. Apoptosis links further to complement C1q because C1q has been described to bind to apoptotic cells and to promote the clearance of apoptotic cells. Finally, C1q
has a strong link back to SLE, because homozygous C1q deficiency is the strongest disease susceptibility gene for the development of SLE (3, 4). However, most patients with SLE have no primary C1q deficiency. Nevertheless, there are indirect links from C1q to SLE: in particular, during flares a substantial number of SLE patients develops hypocomplementemia of the components of the classical pathway of complement, including C1q. Second, C1q is well known to be deposited in affected tissues (6, 7). Last, autoantibodies against complement C1q are frequently found in patients with SLE with a strongly increasing prevalence in case of renal flares (28). By the demonstration that anti-C1q specifically recognize C1q bound on early apoptotic cells, we could demonstrate that binding of C1q to Igs/IC indeed leads to conformational changes of the molecule. The lack of binding of anti-C1q to these neoepitopes suggests that conformational changes of C1q are complex and strongly dependent on the nature of the ligand.

The observation that anti-C1q specifically recognize C1q bound on early apoptotic cells, as shown by FACS analysis and confocal microscopy, is not surprising. Apoptotic cells have already been described to be a potential source of autoantigens in SLE. For example, nucleosomes that are considered a main autoantigen in human and murine lupus are primarily generated by apoptosis (46, 47). Furthermore, intracellular and nuclear Ags as targeted in SLE were shown to be translocated to the cell membrane and modified during apoptosis (18). In addition, the injection of apoptotic cells into healthy mice could promote the development of anti-nuclear autoantibodies (19). C1q acts as a bridging molecule between apoptotic cells and macrophages and stimulates the process of ingestion (23). In the context of an altered clearance of apoptotic cells due to mechanisms that are independent of C1q, C1q itself exposing unusual conformational epitopes could become antigenic and give rise to the production of anti-C1q autoantibodies. However, to date there has not been direct evidence for the hypothesis that prolonged exposure of C1q on incompletely cleared apoptotic cells indeed is the origin of the autoimmune response in SLE. The observation that anti-C1q are specifically directed to C1q bound on early apoptotic cells is now providing first evidence for this hypothesis. Even more, the exceptional expression of neoepitopes by C1q and binding characteristics of anti-C1q strongly support the hypothesis that in general early apoptotic cells are the major target of the autoimmune response in SLE.
We also observed binding of anti-C1q Fab autoantibodies to late apoptotic/necrotic cells that was independent of the presence of C1q, an observation for which we have no explanation other than that cells undergoing late apoptosis or necrosis generate various new epitopes, and therefore, this binding might be unspecific. However, we cannot exclude a specific binding of anti-C1q to late apoptotic/necrotic cells targeting structures that remain to be identified. Furthermore, it is possible that C1q-dependent binding of anti-C1q to late apoptotic/necrotic cells had been masked by abundant binding of anti-C1q in the absence of C1q. Because to date the occurrence of late apoptosis has not been demonstrated in vivo, in contrast to early apoptotic cells, and because early apoptosis naturally precedes the occurrence of late apoptotic cells, early apoptotic cells are more likely to be the real target of the autoimmune response in SLE. However, we think that the binding of C1q with the consecutive recognition by anti-C1q occurs in an advanced stage of early apoptosis. In line with our observations, it has recently been shown that a subset of SLE patients produces autoantibodies against a Ro 60 apotope that is exposed on the surface of early apoptotic cells. This immune response was shown to be specific for SLE, because the Ro 60 apotope was almost absent in patients with primary Sjögren’s syndrome (48). In contrast to Abs against the Ro 60 apotope, anti-C1q autoantibodies were shown to strongly correlate with disease activity and are directed against a highly functional molecule. The ability of C1q to sense multiple signals due to its multimeric structure together with the observations that C1q facilitates the clearance of apoptotic cells suggests a central role for C1q in apoptotic cell clearance, and thus in immune tolerance (49, 50). The role of C1q as an actor of immune tolerance has been reinforced by the discovery of its involvement in the biology of dendritic cells (DC) (51, 52). It was shown that C1q is a potent modulator of DC, resulting in cells characterized by an impaired capacity of cytokine production and an impaired up-regulation of costimulatory molecules, leading to a limited T cell response. It was proposed that C1q regulates the threshold of DC activation and thereby prevents hyperactivation of the overall immune response. As a consequence of our findings, the binding of anti-C1q autoantibodies to C1q on early apoptotic cells might alter the clearance of apoptotic cells and enhance the underlying pathogenic mechanisms, misleading DC to a proinflammatory immune response against apoptotic cells.

However, the clinical observation that anti-C1q strongly correlate with the occurrence of severe lupus nephritis, but not with other organ manifestations, remains to be elucidated. Because C1q-deficient mice developed a lupus-like disease with accumulation of apoptotic bodies in the glomeruli, but not in other organs (53), one can speculate that glomeruli are particularly sensitive to defects in the clearance of apoptotic cells, and therefore might be the loci of predilection for the mechanisms mentioned before. Ongoing studies will have to elucidate the consequences of anti-C1q targeting C1q on early apoptotic cells.

In conclusion, links from SLE to apoptosis, from apoptosis to C1q, and from C1q back to SLE are well established. Demonstrating that anti-C1q specifically bind to C1q bound on early apoptotic cells, our data additionally provide a direct link between all three, SLE, C1q, and apoptosis. Furthermore, the exceptional expression of neoepitopes by C1q and binding characteristics of anti-C1q seem to identify early apoptotic cells as a major target of the autoimmune response in human SLE.

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Disclosures

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