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Autoantibodies against C1q in Systemic Lupus Erythematosus Are Antigen-Driven[1]

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Autoantibodies against complement C1q (anti-C1q Abs) were shown to strongly correlate with the occurrence of severe nephritis in patients with systemic lupus erythematosus (SLE), suggesting a potential pathogenic role by interfering with the complement cascade. To analyze the humoral immune response against C1q at the molecular level, we screened a bone marrow-derived IgGκ/IgGλ Fab phage display library from a SLE patient with high anti-C1q Ab titer against purified human C1q. Six Fabs that exhibited strong binding to C1q in ELISA were isolated. The anti-C1q Fabs recognized neoepitopes that were only exposed on bound C1q and not present on soluble C1q mapping to different regions of the collagen-like region of C1q. Analysis of the genes encoding the variable H and L chains of the IgG-derived anti-C1q Fab revealed that all the variable H and L chain regions were highly mutated, with nucleotide and amino acid homologies to the closest germline in the range of 71–97% (average 85 ± 4) and 72–92% (average 88 ± 6), respectively. In addition, the variable region of the Fabs exhibited high replacement to silent ratios. The six anti-C1q Fabs were shown to be of high affinity, with a K_d ranging from of 8.4×10^{-8} M to 1.4×10^{-7} M, comparable to an antiviral immune response. Our data underlines the notion that the development of anti-C1q Abs in SLE is the consequence of an Ag-driven, affinity-matured immune response. Those anti-C1q Fabs are unique tools to address how complement C1q is implicated in the pathogenesis of SLE. *The Journal of Immunology,* **2009, 183: 8225–8231.**

An important hypothesis of the pathogenesis of systemic lupus erythematosus (SLE)[3] assumes that the disease is driven by a defective clearance of dead and dying cells that could become antigenic and elicit autoimmunity (1–5). The potential mechanism is based on 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 (6–7). Lupus-prone mice were shown to have an intrinsic impairment in apoptotic cell uptake (8) and macrophages derived from the peripheral blood of SLE patients also show defective uptake of apoptotic cells (9). In addition, a number of lupus autoantigens in SLE were located on the surface of apoptotic bodies and apoptotic blebs (10). Therefore, apoptotic cell debris could be the source of autoantigens in SLE. Indeed, the injection of apoptotic cells into healthy mice could induce the production of autoantibodies (11) that were directed against typical lupus Ags, including anti-nuclear, anti-DNA, and anti-cardiolipin Abs.

In humans, the complement system has been shown to play an important role in the clearance of apoptotic cells and deficiency of one of the early components of the classical pathway of complement is strongly associated with the development of SLE. In fact, homozygous deficiency of C1q, the first component of the classical complement pathway, has been characterized as the strongest disease susceptibility gene for SLE development in humans (12), although most SLE patients do not have a primary complement deficiency. Nevertheless, secondary hypocomplementemia of the classical pathway is a frequent finding in SLE patients and often associated with the occurrence of autoantibodies against C1q (anti-C1q Abs) (13–14). Although anti-C1q Abs are known to occur in a number of distinct autoimmune disorders, they are best described in patients with SLE (15), wherein the occurrence of anti-C1q Abs was shown to strongly correlate with active renal disease (16–20), suggesting that the anti-C1q Abs are necessary, but not sufficient, for development of a proliferative lupus nephritis (18–19, 21–24). In mice, Trouw et al. (25) showed that anti-C1q autoantibodies deposit in glomeruli together with C1q, but induced overt renal inflammation only in the context of glomerular immune complex disease.

The origin of anti-C1q Abs remains to be identified, although impaired clearance of apoptotic material suggests the possibility that C1q binding to the surface of apoptotic bodies renders it antigenic, similar to nuclear components that are normally not exposed to the immune system. In line with this hypothesis is the finding that anti-C1q Abs are directed against a neo-epitope on the collagen-like region (CLR) that is only exposed on bound C1q. Prolonged exposure of this new epitope to the immune system, e.g., on the surface of incompletely cleared apoptotic bodies, could incite an autoimmune response against C1q. Recent studies also suggest that anti-C1q Abs are specific and that the occurrence of the Ab might be Ag-driven (18, 26–27). To clarify this issue, we performed a detailed molecular analysis of the human Ab response to C1q.

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[3] Abbreviations used in this paper: SLE, systemic lupus erythematosus; anti-C1q, autoantibodies against C1q; CLR, collagen-like region of C1q; FR, framework region; GH, globular head of C1q; R:S, replacement to silent mutation ratio; VH, variable H chain; VL, variable L chain.

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Materials and Methods

Library construction and panning on C1q

For Ab selection, a phage library generated from total RNA of aspirated bone marrow from an SLE patient was generated as described previously (28, 29) and screened on human purified C1q (a gift from Bühlmann Laboratories, Schönenbuch, Switzerland; >99% pure as judged by SDS PAGE). C1q was coated overnight on ELISA plates at a concentration of 0.5 μg/ml. In brief, phages resuspended in PBS containing 1% BSA were incubated for 2 h at 37°C. Unbound phages were removed by washing 10 times with PBS containing 1% BSA. Bound phages, enriched for anti-C1q bearing surface Fab, were eluted with 0.1 M glycine-HCl buffer (pH 2.2) and immediately neutralized using 2 M Tris-base (pH 9.0). The eluted phages were amplified by infection of *Escheria coli* and superinfected with M13 helper phage. The panning procedure was repeated five times. Phagemid DNA was prepared from the last three panning rounds, and the *gene III* fragment was then removed by restriction digest using enzymes *Nhe*I and *Spe*I followed by religation. The reconstructed phagemid was used to transform XL1-blue cells to produce clones secreting soluble Fabs. As controls, two other bone marrow-derived IgG1κ phage display libraries were panned against C1q, one generated from a healthy individual and one from an HIV-1 seropositive donor. Ab Fabs specific for measles virus were previously isolated from the library of the healthy individual (30) and Ab Fabs specific for HIV-1 gp120 were previously isolated from the library of the HIV-1 donor (31). Further, as a control for the integrity of the HIV-1 Ab library, the same library was also panned against HIV-1 gp120.

Production and purification of anti-C1q Fabs

Fabs were purified from bacterial supernatants by affinity chromatography. *E. coli* containing the appropriate clone were inoculated overnight at 37°C into 100 ml cultures of super broth medium containing ampicillin (50 μg/ml) and tetracycline (10 μg/ml). From this overnight culture, 1 ml was used to inoculate 100 ml of super broth containing ampicillin (50 μg/ml), tetracycline (10 μg/ml), and $MgCl_2$ (20 mM) and then incubated at 37°C with shaking until the OD_{600} was 0.9. Thereafter, protein expression was induced overnight by addition of 1 M isopropyl β-D-thiogalactopyranoside at a final concentration of 1 mM and 4 M cyclic AMP at a final concentration of 0.4 mM. The following day, the cultures were centrifuged at 7000 rpm and the bacterial pellets resuspended in 1× PBS containing 0.5 mM PMSF, filtered, sonicated, centrifuged again at 17,000 rpm, and the supernatant loaded onto a column of goat-anti-human $F(ab)_2$ IgG Ab coupled to protein G CNBr-activated Sepharose 4B (Amersham Biosciences). After washing, bound Fabs were eluted at pH 3.3 using 100 mM citric acid, neutralized with 1M Tris (pH 9.0), and dialyzed against PBS (pH 7.2). The degree of purity of the anti-C1q Fab preparations was confirmed by SDS PAGE analysis (12% Tris Gel; Bio-Rad) and silver staining (Amersham Biosciences). Reactivity with purified human C1q but not with control Ags was confirmed by Western blot analysis and ELISA.

ELISA

C1q specificity. The serum of the SLE patient from whom the phage library was obtained was analyzed for the presence of anti-C1q Abs using a commercially available test (Bühlmann Laboratories) according to the manufacturer's instructions and as described previously (24, 32).

Competition with soluble C1q. To assess the specificity of the anti-C1q Fabs derived from phage display, we screened the purified Fabs in an ELISA against human plate-bound C1q. All six anti-C1q Fabs at a concentration of 0.5 μg/ml were preincubated with either 0, 2, or 4 μg soluble C1q before incubation (estimated molar ratios of Fab to C1q were 1:4.4 and 1:8.8, respectively) on a microtiter plate coated with C1q before further processing. For this procedure, C1q was coated onto Costar microtiter plates in carbonate buffer (0.2 M $NaHCO_3$ and 0.2 M Na_2CO_3), pH 9.6, at a concentration of 0.5 μg/well in a total volume of 100 μl overnight at 4°C. Next, the microtiter plates were washed five times with PBS-0.05% Tween 20 and then incubated with 3% BSA blocking solution for 1 h at 37°C. The purified anti-C1q Fabs, a 1/50 dilution of a control serum from a SLE patient previously shown to have high anti-C1q titers, a healthy control serum, and unrelated Fab b12 (specific for gp120 of HIV-1 (IIIB strain)) were incubated in high salt buffer (1 M NaCl, 1% FCS, 0.05% PBS-Tween 20) with the test Ags for 2 h at 37°C followed by five washing steps with PBS-0.05% Tween 20. High salt conditions were used to avoid cross-reactivity of immune complexes to C1q for the control sera. Detection of bound human Fabs and mouse Ab was conducted with alkaline phosphatase-labeled goat anti-human IgG $F(ab')_2$ Ab (Jackson ImmunoResearch Laboratories) diluted 1/500 (1 μg/ml) in 1% PBS-BSA for 1 h at 37°C and visualized with nitrophenol substrate (Sigma-Aldrich) by reading absorbance at 405 nm.

Cross-reactivity of anti-C1q Fabs. Cross-reactivity of all six human anti-C1q Fabs (0.5 μg/ml) was assessed toward human placental dsDNA (Sigma-Aldrich), collagen type I (Calbiochem), collagen type IV, and BSA (both from Sigma-Aldrich), which were coated onto Costar microtiter plates in PBS at a concentration of 0.5 μg/well in a total volume of 100 μl overnight at 4°C or overnight at 37°C in 1% BSA (DNA), after which the ELISA was performed as described above. Reactivity toward nucleosomes was tested using a commercially available kit from Orgentec Diagnostika and the same secondary Abs as used for the other Ags tested. As control, an anti-C1q-positive SLE serum and a healthy control serum in a 1/50 dilution in high salt buffer and unrelated Fab b12 (0.5 μg/ml) were also tested on those Ags. As positive controls for the different coatings, we tested in the same ELISA SLE patient serum with a high anti-DNA titer, as well as rabbit-anti-human alkaline phosphatase-labeled anti-collagen type I Ab, anti-collagen type IV Ab, and anti-BSA Ab, all of which tested highly positive for their respective Ags (data not shown).

Nucleic acid sequencing

Phagemid DNA was extracted using a DNA extraction kit (Qiagen) and directly sent to Microsynth for sequencing analysis. Sequences were then compared with most homologous germline genes in the NBCI Ig Blast GenBank program (http://www.ncbi.nlm.nih.gov/igblast), IMGT database (http://imgt.cines.fr), and VBLAST (http://vbase.mrc-cpe.cam.ac.uk).

Generation of CLR and globular heads (GH) of C1q

For the generation of CLR, 200 μg of C1q were first dialyzed against sodium acetate buffer (pH 4.45) at 4°C overnight before digestion with 14 μg of pepsin (Sigma-Aldrich) for 20 h at 37°C, as described previously (32). The digest was then centrifuged for 20 min at 35,000 g and the supernatant applied on a size exclusion column (Superdex 200 10/300 GL; Amersham Biosciences) using fast protein liquid chromatography for the separation of CLR.

For the generation of GH, 200 μg of C1q were incubated with 800 μg of collagenase (Sigma-Aldrich) for 16 h at 37°C and centrifuged at 14,000 g for 10 min at 4°C. Separation of GH form collagenase and undigested C1q was then performed using fast protein liquid chromatography and a size exclusion column (Superdex 200 10/300 GL, Amersham Biosciences). The protein concentrations of CLR and GH in the collected fractions were assessed by NanoDrop 1000 spectrophotometer (Witec) at OD_{280} nm and the degree of purity of both protein preparations were confirmed by SDS PAGE (12% Tris gel; Bio-Rad) followed by silver staining (Amersham Biosciences). The reactivity with the six purified human anti-C1q Fabs was tested on ELISA as described above by coatings of similar amounts of C1q, CLR, or GH. Unrelated Fab b12, specific for gp120 of HIV-1 (IIIB strain), served as a negative control.

Western blot analysis

Human purified C1q (1 μg), under nonreducing and reducing conditions (final concentration of 0.1 M DTT), was mixed with 5× sample buffer (Pierce), adjusted to a final volume of 15 μl with distilled water, and boiled for 5 min at 95°C before being loaded on a 12% SDS-PAGE HCl-Tris gel (Bio-Rad). The proteins were then electroblotted onto nitrocellulose membrane (Bio-Rad) and blocked (5% nonfat dry milk in 0.05% Tween 20 PBS) for 1 h before overnight incubation at 4°C with 1 μg/ml purified anti-C1q Fabs in blocking buffer. After repeated washing of the nitrocellulose membrane (6 × 5 min), bound Ab was detected with HRP-labeled $F(ab')_2$ of goat anti-human F(ab′)2-specific Ab (1:5000, Serotec) incubated for 1 h and visualized by chemiluminescent substrate (ECL; Amersham Biosciences) and autoradiographic film (CL-Xposure; Sigma-Aldrich). Control staining of immunoblots omitting the primary Ab was included (data not shown).

Affinity measurements of anti-C1q Fabs by ELISA

CLR was first incubated with the anti-C1q Fabs at a constant concentration until equilibrium was reached. The concentration of free Abs was then determined by an inhibition ELISA. Briefly, CLR was preincubated separately at various concentrations (2×10^{-10} M to 2×10^{-7} M) in carbonate buffer (0.2 M $NaHCO_3$ and 0.2 M Na_2CO_3), pH 9.6, with each of the six anti-C1q Fab at its equilibrium concentration in the range of 2–5 $\times 10^{-7}$ M. After 2 h at 37°C, the mixture was incubated onto a microtiter plate previously coated with 0.5 μg/well CLR (coating as described above for C1q) and blocked with 3% BSA for 2 h at 37°C. After washing, bound Fabs were detected using an anti-$F(ab)_2$ alkaline phosphatase-labeled Ab (Jackson ImmunoResearch Laboratories) and the absorption read at 405 nm. The K_d for each anti-C1q Fab could then be calculated using Scatchard plot analysis (Prism graph version 4.1; GraphPad Software).

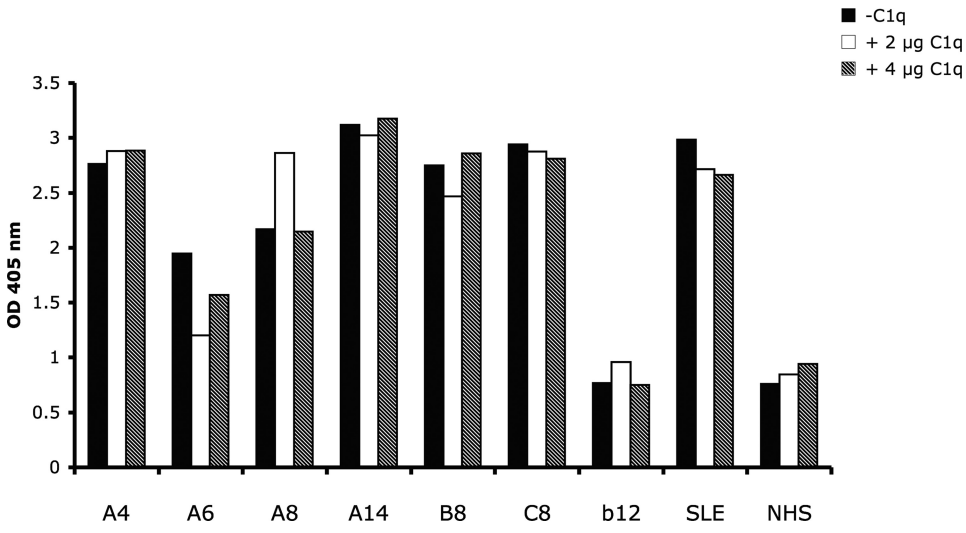

FIGURE 1. Anti-C1q Fabs recognize plate-bound C1q only. Bars represent the acquired absorbance at 405 nm of the six human monoclonal anti-C1q IgG-Fabs (A4, A6, A8, A14, B8, and C8) binding to plate-bound C1q after preincubation with either no (■), 2 (□), or 4 (▨) μg of soluble C1q by competition ELISA. Fabs were tested at a concentration of 0.5 μg/ml under high salt conditions. Binding of the six anti-C1q Fabs were in the range of serum from a patient with SLE, with a high titer of anti-C1q Abs as determined previously and substantially above absorbance values of unrelated anti-HIV-1 gp120 Fab b12 and normal human serum (NHS), which served as positive and negative controls, respectively.

Results

Cloning of human IgG autoantibodies to C1q

To analyze the humoral immune response against C1q, we screened an IgG1κ/IgGλ Fab phage-display library of 4×10^8 members generated from aspirated bone marrow of an SLE patient. The serum was shown to contain high anti-C1q Ab titer (74 U/ml, norm $\leq$15). In concordance with the standard clinical method for determining anti-C1q, the patient's Ab library was screened against purified human C1q coated onto ELISA plates in the presence of 1 M NaCl to avoid low-affinity binding (33). After five rounds of selection, a 1000-fold amplification of the IgGκ phage-display library (2.8×10^6 size) and a 100-fold amplification of the IgGλ Fab phage-display library (0.3×10^4 size) in eluted phages were observed, indicating enrichment for Ag-binding clones. Controls consisted of two bone marrow-derived IgG1κ phage display libraries, one generated from a healthy donor (30) and one from a HIV-1 seropositive donor (33). With the control libraries, no enrichment in eluted C1q specific phages was observed and the phage titers declined with each round of selection. In contrast, when the Ab library from the HIV-1 positive donor was selected on HIV-1 gp120 in parallel, the phage titers increased 10,000-fold (supplemental Table S1).[4]

ELISA screening of supernatants from 60 clones from the fourth and fifth rounds of selection with the SLE library yielded six Fabs that exhibited strong binding to human C1q. As described for serum anti-C1q Abs from SLE patients (33, 34), the anti-C1q Fab binding was specific for plate-bound C1q because preincubation of the purified anti-C1q Fab with excess liquid phase C1q did not lead to any substantial reduction of the binding to plate-bound C1q (Fig. 1).

Specificity of the monoclonal anti-C1q Fabs

To specify the epitope on C1q recognized by human autoantibody Fabs, C1q was digested either with pepsin to obtain the N-terminal CLR or with collagenase to obtain the C-terminal GH region of C1q. The CLR or GH region fractions were then purified over a size exclusion column and coated onto ELISA plates. Interestingly, CLR was recognized equally well as the undigested C1q, with three (A4, A6, and B8) of the six anti-C1q Fabs tested showing an ever higher signal on CLR than on undigested C1q, whereas binding to the GH was not observed for any of the anti-C1q Fabs (Fig. 2*A*). Control sera obtained from healthy individuals showed weak reactivity with C1q or digested fractions of C1q, whereas control serum from a SLE patient recognized C1q and CLR but not GH. Finally, heat-aggregated IgG bound C1q and GH as expected, but not CLR (Fig. 2*A*). To further assess specificity, we tested all six anti-C1q Fabs for reactivity to human placental dsDNA, collagen type I and type IV, BSA, and nucleosomes by ELISA (Fig. 2*B*). As seen in Fig. 2*B*, no cross-reactivity of the tested Ags was observed for any of the anti-C1q Fabs. Absorbances at 405 nm were similar to negative controls, consisting of a healthy serum and unrelated HIV-1-specific Fab b12 tested in the same assay. To determine whether the six human anti-C1q Fabs differ in their recognition of the three similar, but distinct, polypeptide chains of the C1q (A, B, and C), products of three distinct genes aligned in the same orientation (A-B-C) on a 24 kb stretch of DNA on chromosome 1p (35), we performed a Western blot assay (supplemental Fig. S1). The epitope recognized by the six Fabs is at least partly linear and involves conserved epitopes present on at least two of the three polypeptide chains of the C1q molecule.

Human anti-C1q Abs results from an Ag-driven response

To determine whether human Abs to C1q evolved as a result of an Ag-driven immune response, we compared the genes encoding the variable heavy and light chains of the IgG-derived anti-C1q Fabs with the closest germline sequences in the GenBank, IMGT, and VBASE databases (Fig. 3). All the variable H and L chain genes of the anti-C1q Fabs were highly mutated, with nucleotide and amino acid homologies to the closest variable H chain (VH) germline genes in the range of 71–92% (average 81%) and 72–92% (average 82%), respectively (Table I) and to the closest variable L chain (VL) germline genes in the range of 92–97% (average 94%) and 85–91 (average 88%), respectively (Table I). Additionally, the VH and VL chain genes exhibited a high replacement to silent mutation ratio (R:S) for the CDR1 and CDR2 compared with the framework region (FR) 1, FR2, and FR3 (Table I) for all Fabs (with the exception of Fab A8), which is characteristic of an affinity-matured Ab response (36–46). The two Fabs A4 and A14, which exhibited the strongest binding to C1q (Fig. 1), had the highest R:S ratio for the CDRs compared with the other anti-C1q Fabs. Additionally, these clones exhibited a substantially higher R:S ratio for the CDRs compared with their FRs (Table I). Strong binding to C1q was seen when the deduced amino acid homologies to the closest germline were around 83%, such as that seen in anti-C1q Fab A4, A14, and C8 (Fig. 1). The sequence analysis of the H chain also showed that the three Fabs (A4, A14, and B8) had

[4] The online version of this article contains supplemental material.

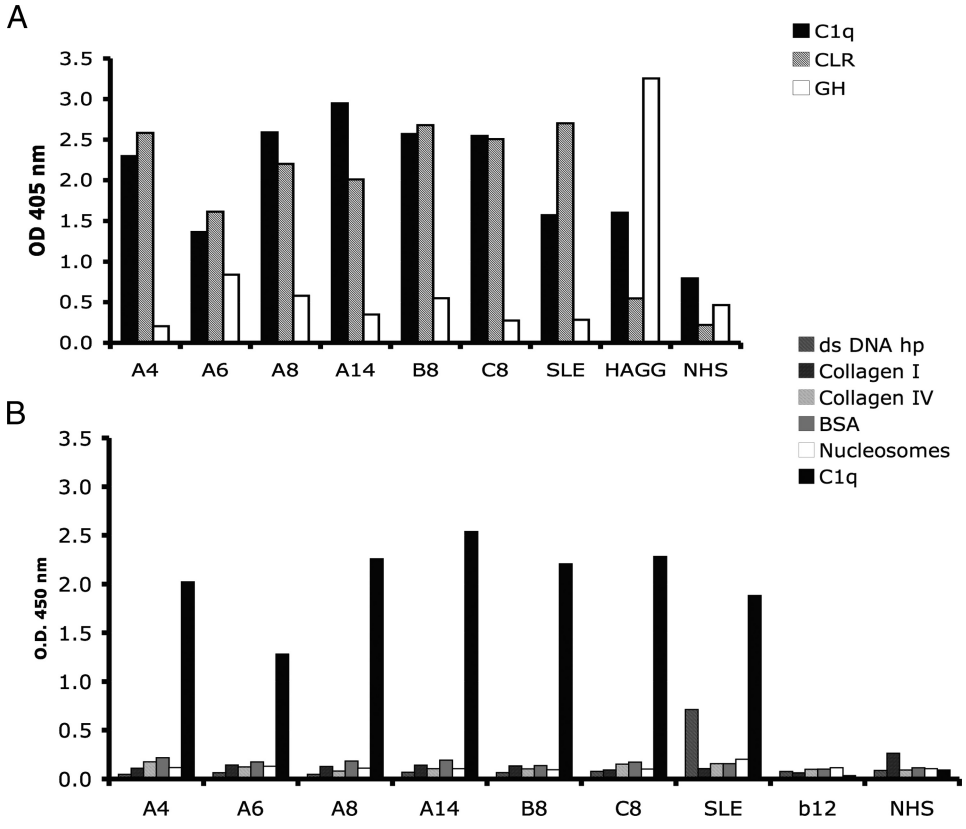

FIGURE 2. Determination of the specificity of the six human monoclonal IgG-Fabs by ELISA. *A*, Bars represent the acquired absorbance at 405 nm for binding of IgG-Fabs (1 μg/ml) to undigested C1q, CLR, and GH. As controls, serum from a SLE patient, heat-aggregated IgG (HAGG) generated from a healthy donor, and normal human serum (NHS) from a healthy donor were used. *B*, Reactivity of the six monoclonal human IgG-Fabs (1 μg/ml) against unrelated Ags (coated at 5 μg/ml), namely human placental dsDNA (dsDNA hp), collagen type I and type IV, BSA, and nucleosomes was assessed by measuring the absorbance at 405 nm by ELISA. None of the anti-C1q IgG-Fabs cross-reacted with the Ags tested and were in the range of the absorbance signal of unrelated anti-HIV-1 gp120 Fab b12 and serum from a healthy donor used as controls. As a positive control, serum from a SLE patient known to be positive for anti-DNA autoantibodies was included in the assay.

closely related sequences, which were likely somatic variants that had evolved from a common ancestor. Interestingly, one anti-C1q Fab A8 had a much higher R:S mutation rate of the FR when compared with the CDRs, which is unusual for an Ab exhibiting high affinity for its Ag. Therefore, mutations in the CDR must be the major contributor to the C1q affinity.

The junction JH and JL genes of the anti-C1q Fabs were also highly mutated and exhibited nucleotide homologies to the closest

A

Heavy chain

Clone	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
VH2-26*01	LKESGPVLVKPTETLTLTCTVSGFSLS	NARMGVS	WIRQPPGKALEWLA	HIFSN DE KSYSTSLKS	RLTIS KD TSKSQVVLTMTNMDPVDTATYYCAR		
A4	LKESGPVLVKPTDTLTLTCTVSGFSLS	NA I IGLT	WIPQPPGKAREWLA	HPKSRDVKGYRTSLKS	RLAIAKYASKSQVVLTMTNMDPVDTACYYCAR	KYPRDDSILTATYMDV	WGQGTLVLFSS
A14	LKESGPVLVKRTETLTLTCTVSGFSLS	NPI NGLS	WIPQPPGKAREWLA	HPKSRDVKGYRTSLKS	SLAIAKYASKSRVVLTMTNMDPVDTACYYCAR	KYPRDDSILLSGRYMAE	WGQGTLVLFSS
B8	LKESGPVLVKPTETLTLTSTVSAFSLS	NAI FGEC	WIRQPPGKALEWLA	HIFSR DE KSYSTFLTS	RLTIP KD TSKTQVVLTMTNMDRVETATYYCAR	KCPGEDYIPIGRYMAV	WGQGTLVLFSS
VH2-5*01	LKESG PTLVK PTQLTLTCTFSGFSLS	TSGVGVG	WI RQPPGKALEWLA	LIYWN DDK RYSPSLKS	RLTITKDTSKNQVVLTMTNMDPVDTATYYCAH		
A6	LRDSVPTLLQPRQLTLTWTFCGFSLS	TSAVAVG	WMRHPPGKALE LLA	LFYWNHDYRYRPSLMS	RLTITKDTSKNQVVPTKTTMDPADIATFYSAP	SLVFTAAFNALDT	WGQGIVVRVFS
VH3-20*01	LVESGGGVVRPGGSLRLSCAASGFTF D	DYGMS	WVRQAPGKG LEWVS	GINWNGGSTGYAD SVKG	RFTISRDNAKNSLYLQMNSLRAEDTALYHCAR		
A8	LVESGGGEVPPGGSLRLSFGASAFTYD	DYGIS	WVPQARVTVLEWVS	GISWSRGCTGYADFEMG	RFSICSEKVKNSLYLHLNTRRAEVTGFYHCAR	PRSSGAYYKPLD	WGQGITVNVFS
VH4-4*07	LQESGPGL VKPSETLSLTC VSGGSIS	SYYWS	WIRQPAGKGLEWIG	RIYTSGSTNYNPSLKS	RVTMSVDTSKNQFSLKLSSVTAADTAVYYCAR		
C8	LQESGPGQVMPSETLSLTCLSGGSIT	SYYSS	WIRLPAGKGLEWIG	RNYSCGITGFNPSLKS	RVTISVDTSKNQYSQKLSCVAAADTAVYYCAR	DKGSEYSFDP	WGQGTLVTVSS

B

Light chain

Clone	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
Vk1D-13*02	LTQSPSSLSASVGDRVTITC	RASQGISSALA	WYQQKPGKAPKLLIY	DASSLES	GVPSRFSGSGSGTDFTLTISSLQPE DFATYYC		
A4	LTPSPSSLSASVGDRVTITC	RASEGISSGLA	SYQQKPGKAPKLLIY	DGSIMES	GVPSRFSGSASGSYFTVTISSLQPEDFATYYC	HQYGASLVS	FGGGKTLEIKRT
A6	LTQSPSSLSASVGDRVTITC	RSSEAISSALA	WNQLKPGKAPKLLIY	DGSSLES	GVPSRFSGSAFGRDFTLTISSLQPEDFAAYYC	QQGNSPFPK	FGPGTVVDIKR
A14	LTPSPSSLSASVGDRVTITC	RASEAISSALA	WNQQKPGKAPKLLIY	DGSSLES	GVPSRFSGSGSGRDFTLTISSLQPEDFAT YYC	QVYGQSSPVL	FGQGTKLEIKTR
B8	LTQFPGSLSASVGDRVTITC	PVNLGISSGLA	WKQQIPGKAPKLLIY	DACSLES	GVPSRFSGSGSGTDFTLSFCTLQREDFATYYC	QYYGGSSYTF	FGQGTKVLGRT
Vk2-24*01	MTQTPLSSPVTLGQPASISC	RSSQSLVHSDGNTYLS	WLQQRPGQPPRLLIY	KISNRFS	GVPDRFSGSGAGTDFTLKISRVEAEDVGVYYC		
A8	MTQSPLSSPVTLGQPASISC	TCTLSLVHSDGNTYLS	WLQQSPGHPPRLLIY	KISNRFS	GVPDSFSGSGAGTEFTLKISTEEAEDVGVYYC	QQYGDSPLFK	FGQPGTVEITR
VL11-55*01	LTQPPSLSASPGATARLPC	TLSSDLSVGGKNMF	WYGQQKPGSSPRLFLY	HYSDSDKQLGP	GVPSRVSGSKETSSNTA FLLISG LQPEDEADYYC		
C8	LTQRRSLSASPGATARLPW	TLISHLTVGGKNVF	WYLQQKPGSSPRLFLY	HYSDSEKQLGP	GVPSRVSGSMETSSNTAYLLMSALQPEDEADYYC	QYYGGSSYT	FGQGTKVLEIKTR

FIGURE 3. Alignment of the amino acid sequence of each anti-C1q variable heavy (*A*) and variable light (*B*) domains of the anti-C1q Fabs to their closest germline sequence. All six anti-C1q IgG Fabs cloned from a SLE patient were highly somatically mutated. Residues believed to have arisen from somatic mutations (deduced from comparison to the closest germline sequence) are in red, indicating an amino acid that is altered in the anti-C1q Fab protein sequence. Fabs are grouped according to the closest V gene alignment.

Table I. *Comparison of the nucleotide and deduced amino acid sequences of the anti-C1q Fabs with the closest germline sequences for the VH and VL chains*

Clone	Closest V Germline	Amino Acid Homology (%)	Nucleic Acid Homology (%)	R:S Ratio FR	R:S Ratio CDR	Closest D Germline	Closest J Germline
VH chain							
A4	VH2–26*01	86.5	91.5	8:3 = 2.6	9:1 = 9	DH2–24*01	JH6*01
A14	VH2–26*01	85.9	90.6	4:6 = 0.8	9:1 = 9	DH6–19*01	JH4*03
B8	VH2–26*01	92.4	91.8	4:8 = 0.5	5:2 = 2.5	DH3–10*02	JH6*01
A6	VH2–5*01	78.2	70.6	18:4 = 4.5	8:1 = 8	DH3–16*02	JH6*02
A8	VH3–20*01	71.7	88.4	26/2 = 13	9:2 = 4.5	DH1–26*01	JH6*01
C8	VH4–4*07	85.3	92.0	16:6 = 2.6	7:2 = 3.5	DH5–24*01	JH5*02
VL chain							
A4	Vk1D-13*01	91.0	97.0	6:2 = 3	5:1 = 5	NA	JkV3D*01
A14	Vk1D-13*01	87.0	95.7	6:2 = 3	4:1 = 4	NA	Jk2*04
B8	Vk1D-13*01	90.0	95.5	2:4 = 05	3:1 = 3	NA	Jk2*02
A6	Vk1D-13*01	85.5	94.5	9:3 = 3	6:2 = 3	NA	Jk3*02
A8	Vk2–24*01	89.8	95.5	7:4 = 1.7	4:1 = 4	NA	Jk3*01
C8	VL11–55*01	89.0	92.0	8:4 = 2	6:2 = 3	NA	JL1*01

NA, Not applicable.

JH and JL germline genes in the range of 60–72% (average 66%). The identification of the closest D segment proved even more difficult due to significant somatic modifications in the range of 60–80% (average 70%). The closest J and D germline genes alignments, as presented in Table I, could only be found by using less stringent conditions because only a few nucleic acids or short stretches of nucleic acids were homologous to the anti-C1q Fabs (no significant score) when analyzed with the different Ig databases.

Comparison of the relative affinity of the panel of anti-C1q Fabs

Autoantibodies involved in an active immune response generally exhibit high affinity for their Ag. To evaluate the affinity of the anti-C1q Fabs, we tested all six anti-C1q Fabs in an inhibition ELISA wherein the binding of all Fabs to plate-bound CLR was inhibited by preincubation with increasing concentrations of soluble CLR. The absorbance at 405 nm represents the concentration of free anti-C1q Fabs able to bind to plate-bound CLR used to calculate the percent inhibition of maximum binding (Fig. 4). As described by Friguet et al. (47), this inhibition ELISA allows calculation of the K_d using Scatchard plot analysis. Affinities assessed in such a way were very similar for all six anti-C1q IgG Fabs in the range of 8.4×10^{-8} M to 1.4×10^{-7} M (individual K_d values are depicted in Table II). Although those results are relative affinities, it is important to note that the strength of the immune response of those autoreactive monoclonal anti-C1q Fabs is in the range of an anti-viral immune response (10^{-8} M). Taken together, the high degree of somatic mutation, high R:S ratios, and interclonal variants and the affinities, as assessed by Scatchard plot analysis and sustained high salt conditions, strongly suggest that the described human monoclonal anti-C1q Abs are derived from B cells involved in an Ag-driven, affinity-matured immune response.

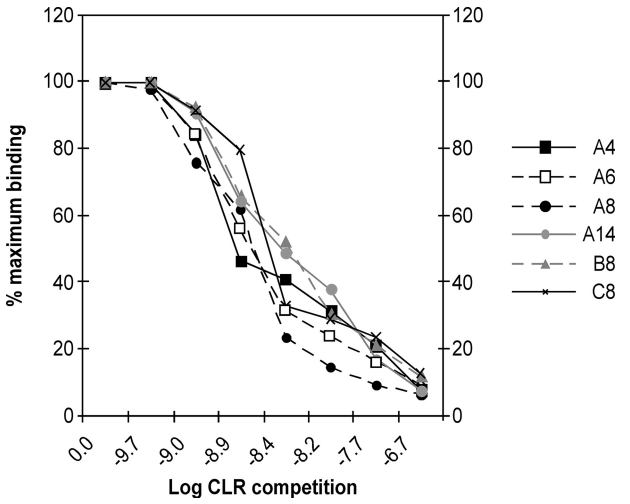

FIGURE 4. Relative affinities of the panel of six anti-C1q Fab toward CLR as measured by inhibition ELISA. Anti-C1q IgG Fab at a constant concentration of 2.5×10^{-7} M were preincubated without CLR (100% maximum binding) or with serial dilutions of CLR ($2–10 \times 10^{-7}$ M) before adding to microtiter plate coated with CLR. By measuring the absorbance at 405 nm, we calculated the amount of inhibition of binding to plate-bound CLR with increasing amounts of soluble CLR concentration. As an internal control, absorbance of the anti-HIV-1 Fab b12 on recombinant gp120 was measured the same way and its affinity recalculated. Affinities of the six human anti-C1q IgG-Fabs were in the range of 8.4×10^{-8} M to 1.4×10^{-7} M as assessed by Scatchard plot analysis. Individual values for each anti-C1q Fab are depicted in Table II.

Binding of anti-C1q Abs to C1q relays not only on charge-charge interactions

The binding of anti-dsDNA Abs to dsDNA has been found to be associated with the enrichment of arginine residues in the CDR3 of the H chain of these Abs. Because C1q is a basic protein, we analyzed whether the Fabs or each of their CDR and FR of the six anti-C1q Fab Abs were enriched for acidic residues to determine whether binding of the anti-C1q Fab Abs to C1q relied only on charge-charge interactions. Interestingly, no enrichment of acidic residues was observed. Further, the six anti-C1q Fab Abs exhibited a wide range of isoelectric points (3.1- 9.5) for the entire Fab domain as well as for the individual CDR and FR (Table S1). Additionally, no correlation between the individual affinity constant of the six anti-C1q and their respective isoelectric points was observed. Together, these

Table II. *Relative affinities (K_D) as measured by Scatchard plot analysis*

Clone	K_D values
A4	5.16×10^{-8}
A14	8.35×10^{-8}
B8	4.72×10^{-7}
A6	1.40×10^{-7}
A8	7.80×10^{-7}
C8	5.20×10^{-7}

data indicate that the binding of the anti-C1q Fab Abs to C1q is not based on charge-charge interactions alone, but that conformational structures, such as protein motifs, likely also play a role.

Discussion

In this study, we have shown that human anti-C1q Abs derived from the bone marrow of a SLE patient arise as a result of an Ag-driven immune response. This observation supports the view that complement C1q is implicated in the pathogenesis of the disease. This hypothesis is primarily based on the observation that most patients with C1q deficiency develop a lupus-like syndrome (15, 48), with homozygous C1q deficiency being the strongest susceptibility gene for the development of SLE despite the fact that most SLE patients do not show a primary complement deficiency. Nevertheless, secondary hypocomplementemia is a frequent finding in SLE patients due to the consumption of early components of the classical pathway of complement, including C1q (13–14, 49). The reason for this consumption is not fully understood, but may partially be explained by the detection of anti-C1q Abs in ~20–50% of unselected SLE patients and more than 95% of patients with severe lupus nephritis. Although anti-C1q cannot account for hypocomplementemia in all patients with SLE, there is a strong correlation between the occurrence of these autoantibodies and hypocomplementemia (13, 23, 50). An increasing number of studies suggest a pathogenic role of anti-C1q Abs in SLE, particularly in the development of proliferative lupus nephritis (24, 51), because anti-C1q Abs strongly correlate with renal flares (52).

In contrast to many other autoantibodies described in SLE, anti-C1q Abs are directed against a highly functional molecule. It has been shown that C1q, as the first molecule of the classical complement pathway, plays an important role in the clearance of immune complexes and apoptotic cell debris (11–12, 53). Anti-C1q Abs may alter the physiological role of C1q by occupying important binding sites for C1q receptors and thus might interfere with the process of cell lysis and uptake of immune complexes and apoptotic bodies.

To characterize the anti-C1q Abs response on a molecular level, we screened a human IgGκ-IgGλ Fab phage-display library and retrieved six anti-C1q Fabs. All Fabs showed the typical characteristics reported previously (13, 18, 26, 33–34, 50, 54): high specificity even under high salt conditions for the CLR of plate-bound C1q but not for soluble C1q. These binding characteristics are thought to occur due to the expression of neoepitopes on C1q in its bound form (51, 55). Concordant with this hypothesis are our findings in Western blot assay wherein different C1q polypeptide chain recognition patterns were observed for the six anti-C1q Fabs tested, suggesting involvement of different epitopes on C1q (supplemental Fig S1). The fact that we could generate multiple specific Abs to C1q from the SLE patient library, with high affinities and within the range of other human Abs as well as human anti-viral Abs (40, 56), provides evidence for an immune response actively driven by bound C1q. Bone marrow was used as source for the phage libraries, as it is a major repository for plasma cells producing Abs, broadly reflecting the repertoire found in serum (57, 58). Unless the donor has reasonable numbers of specific plasma or B cells and corresponding serum Abs titers, specific high-affinity human Abs cannot be cloned from the small immune libraries generated from patients like the one we have screened. As indicated by the comparison of the genetic diversity of the six anti-C1q Fabs (Table I), we are confident that we have selected a representative panel of anti-C1q Abs from the bone marrow of this SLE patient. The anti-C1q Fabs showed extensive somatic mutation, with high R:S ratios in the CDRs. As shown previously for autoantibodies of pathogenic relevance, the immune response is characterized by a high degree of somatic hypermutation in the Ig genes during B cell maturation (33, 59, 60). Autoantigens, as has been shown for DNA, can trigger B cell responses by simultaneously activating B cell Ag receptor and TLR signaling pathways leading to potent B cell responses such as B cell maturation (10). Thus, the high R:S ratio and overall somatic mutations of the anti-C1q Fab clearly show that they were produced as a result of an affinity-matured immune response.

It has been shown that the appearance of self-reactive Abs in SLE precedes renal flares but the point in the B cell pathway where tolerance is first broken has not been defined. In line with the findings of Yurasov (61), who found that the Ag-driven IgM immune response toward C1q in SLE patients seemed to be biased toward use of a restricted VH repertoire, we found IgG Fabs belonging to the gene families VH3, VH4, and VH2. It is interesting to note that four of the six anti-C1q IgG Fabs belong to the VH2 gene family, which has rarely been reported in that context. Along with the observed increased somatic hypermutation, this is consistent with findings that Ag selection pressure along with VL receptor editing might shape the Ig repertoire differently in autoimmune disease compared with healthy controls (59, 62–64). Nevertheless, the factors that lead to this apparent abnormal B cell tolerance accounting for B cell maturation and affinity selection remain to be explored.

In conclusion, our analyses of human bone marrow-derived anti-C1q Fabs generated from the B cell repertoire of a SLE patient suggest that the development of anti-C1q Abs is the consequence of an Ag-driven immune response. This observation supports the hypotheses that both complement C1q and the process of affinity maturation of autoantibodies in general are implicated in the pathogenesis of the disease. The generated monoclonal Fabs will be an excellent tool for further studies on the epitope and the role of anti-C1q Abs in the pathogenesis of SLE.

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Disclosures

The other authors have no financial conflict of interest.

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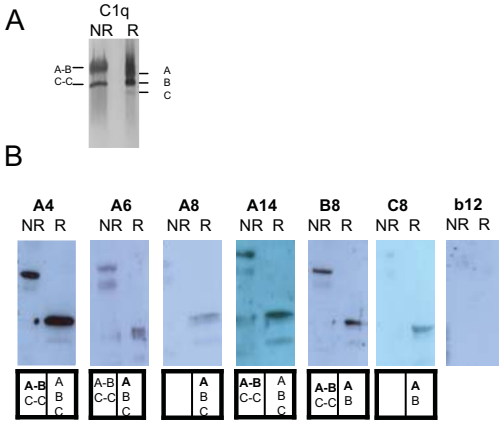

Fig. SI. Epitope characterization of the human anti-C1q Fabs. All anti-C1q Fab fragments recognized at least 2 of the 3 polypeptide chains of C1q. (A) SDS-PAGE gel separation of full-length C1q showing the similar but distinct patterns of the 3 polypeptide chains A, B and C under non-reducing (NR) and reducing (R) conditions after silver staining. The C1q molecule is composed of 3 similar, but distinct, polypeptide chains, A, B and C, which are the product of three distinct genes aligned in the same orientation in the order of A-B-C on chromosome 1p. Under non-reducing conditions A-B and C-C chain dimers are formed, whereas under reducing conditions all three polypeptide chains are separated. (B) Purified anti-C1q IgG-Fab fragments (2µg/ml) were tested for binding to C1q under non-reducing (NR) and reducing (R) conditions in Western blot analysis. The table below the blots summarizes the polypeptide chain recognized, bold indicating a strong recognition signal. Interestingly, all the anti-C1q Fabs recognized at least two different polypeptide chains. Fabs A4, A6 and A14 recognized all three polypeptide chains under both non-reducing and reducing conditions, whereas the binding seemed to be weaker for Fab A6 compared to Fab A4 and A14. Fab B8 recognized the A-B dimer strongly, but the C-C dimer only weakly under non-reducing conditions, whereas only the A and B chain bound under reducing conditions. Fabs A8 and C8 bound only the A and B under reducing condition and not the C polypeptide chain. These observations indicate that the epitope recognized by the 6 Fabs is at least partly linear and involves conserved epitopes present on at least two of the three polypeptide chains of the C1q molecule.

Table S1. pI values of the different human anti-C1q Fabs and each of their CDR and FR regions.

		Fabs	CDR1	CDR2	CDR3	FR1	FR2	FR3
Heavy-chain	A4	4.1	6	10.8	4.1	6.3	10.9	8.9
	A6	3.1	6.1	9.4	3.1	9.1	10.1	9.5
	A8	9.5	3.1	4.1	9.5	3.8	6.3	9.9
	A14	6.9	6.1	10.9	6.9	8.9	10.9	8.9
	B8	6.3	3.3	7.7	6.3	7.1	10.1	9.5
	C8	3.7	5.8	9.7	3.7	3.1	10.1	8.9
Light-chain	A4	4.4	11.1	3.0	7.7	6.1	10.2	3.7
	A6	4.4	1.9	3	10.1	6.1	10.4	4.3
	A8	3.0	4.9	11.4	6.6	5.3	9.8	3.4
	A14	4.4	6.9	3	5.9	6.1	10.4	4.3
	B8	4.3	6.1	3	5.8	6.1	10.4	4.3
	C8	9.3	10.9	5.2	5.8	12.7	10.4	3.5

Table S2. Titers of eluted phage of consecutive rounds of selection.

	HIV-C1q (1)	HIV-gp120 (+1)	NC-C1q (2)
	1.0×10^8	1.0×10^8	8.8×10^6
1st	3.2×10^4	4.0×10^5	3.6×10^4
2nd	2.6×10^3	1.6×10^7	1.3×10^2
3rd	< 1	1.9×10^9	$< 1 \times 10^1$
4th	nd	1.6×10^9	nd

HIV-C1q designates that the HIV-1 patient-derived phage library was panned on C1q, **HIV-gp120** designates that HIV-1 patient-derived phage library was panned on gp120, **NC-C1q** designates the healthy individual-derived phage library was panned on C1q. nd=not deterimined