

On-chip Complement Activation Adds an Extra Dimension to Antigen Microarrays*

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Antibody profiling on antigen microarrays helps us in understanding the complexity of responses of the adaptive immune system. The technique, however, neglects another, evolutionarily more ancient apparatus, the complement system, which is capable of both recognizing and eliminating antigen and serves to provide innate defense for the organism while cooperating with antibodies on multiple levels. Complement components interact with both foreign substances and self molecules, including antibodies, and initiate a cascade of proteolytic cleavages that lead to the covalent attachment of complement components to molecules in nanometer proximity. By refining the conditions of antibody profiling on antigen arrays we made use of this molecular tagging to identify antigens that activate the complement system. Antigen arrays were incubated with serum under conditions that favor complement activation, and the deposited complement C3 fragments were detected by fluorescently labeled antibodies. We used genetically C3-deficient mice or inhibition of the complement cascade to prove that the technique requires complement activation for the binding of C3 to features of the array. We demonstrate that antigens on the array can initiate complement activation both by antibody-dependent or -independent ways. Using two-color detection, antibody and complement binding to the relevant spots was measured simultaneously. The effect of adjuvants on the quality of the immune response and binding of autoantibodies to DNA with concomitant complement activation in the serum of mice suffering from systemic autoimmune disease was readily measurable by this new method. We propose that measurement of complement deposition on antigen microarrays supplements information from antibody binding measurements and provides an extra, immune function-related fingerprint of the tested serum. *Molecular & Cellular Proteomics* 6: 133–140, 2007.

The immune system is traditionally divided into innate and adaptive arms. Although these two arms are highly interconnected and mutually interactive, we generally regard antibod-

ies as components of the clonal adaptive immune response, whereas the complement system functions as a non-clonal innate mechanism for maintaining body integrity. Antibodies can be beneficial or deleterious: those against infectious agents can protect from disease, whereas antibodies against self molecules can induce autoimmune reactions. Because antibodies have identifiable target molecules they became the cornerstone for diagnosing states of disease. In the last decade the importance of innate immunity was reconsidered, revealing that it has decisive roles both at the initiation and effector phase of the immune response (1, 2). Yet modern high throughput technologies have not yet been applied for the analysis of the complement system.

Current multiplex assays for profiling serum immune reactivity utilize only antibody binding as readout. Antigens spotted in microarray format have been used for measuring serum antibodies against microbial components (3, 4) or whole microbes (5), allergens (6–8), and self structures (9–13). These assays are useful for simultaneously identifying several antigen-antibody interactions and therefore help to draw a more complete picture about the immune status of the tested individual. This is, however, a still picture because the presence of an antibody with a particular specificity does not necessarily mean disease or immunity to infection. Certain aspects on the level of a particular antibody molecule, like affinity and isotype, and others on the level of the organism, like overall concentration of antibodies against a target, relative concentration of antibodies with different isotypes, and structural arrangement of the recognized epitopes on the target, together determine the ultimate functional effects of antibodies. One of these functional effects is the initiation of complement activation.

The complement system comprises about 30 proteins, including soluble and membrane-bound components. Certain recognition molecules are able to initiate the complement cascade of proteolytic cleavages, leading to the generation of complement protein fragments with various biological activities. Three pathways of complement activation have been described: the classical, lectin, and alternative pathways. The classical pathway can be initiated by antigen-bound antibodies (14) and other molecules that bind C1q, like C-reactive protein or serum amyloid P protein (15). The lectin pathway relies on the mannose binding lectin and ficolins, molecules that recognize carbohydrate patterns (16–18). The alternative

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pathway is capable of autoactivation; therefore surfaces that lack or are unable to bind complement regulatory proteins promote its activation (19). Importantly all three pathways converge at the point of complement C3 cleavage and activation, a point where an amplification loop insures the generation of sufficient amounts of C3 fragments. Complement component C3 is additionally one of the most abundant serum proteins after albumin and immunoglobulins. Thus, whichever pathway initiates the cascade, C3 fragments will be generated with high efficiency. The initial products of C3 cleavage are a small peptide, C3a, with inflammatory properties and a bigger fragment, C3b, with proteolytic activity as part of a complement convertase enzyme complex. Upon cleavage, an extremely reactive thioester group of the C3 molecule becomes exposed (20) and reacts with a nearby hydroxyl or amine group (21). As a result, the molecules at the site of complement activation will be covalently tagged with the C3 activation and degradation fragments C3b, iC3b, and C3d.

To obtain additional, function-related information from antigen arrays we have developed a reverse immunoassay technique that allows the detection of complement activation by the features of the array. This technique takes advantage of the deposition of complement C3 fragments to the molecules that initiate the complement cascade. We have characterized the system with respect to sensitivity and specificity and worked out proper controls to allow for semiquantitative measurements. We show that the technique, in combination with antibody profiling measurements, can be used to provide additional functional information beyond antibody binding.

EXPERIMENTAL PROCEDURES

Collecting Serum Samples for Complement Deposition Measurements—BALB/c and C57/B6 mice were bred at the animal facility of the university and used at the age of 6–8 weeks. Serum from MRL/lpr mice was generously provided by Nikolina Mihaylova (Bulgarian Academy of Sciences, Sofia, Bulgaria); serum from C3-deficient (22) and JHD animals were a kind gift from Matyas Sandor (University of Wisconsin-Madison). For obtaining immune sera groups of three mice were immunized by injecting 100 μ g of antigen alone or mixed with complete Freund's adjuvant (Sigma) in a total volume of 200 μ l, 100 μ l subcutaneously into the base of the tail and 100 μ l intraperitoneally. After 2 weeks the immunization was repeated according to the same regimen except that incomplete adjuvant was used. Mice were sacrificed after 4 weeks by exsanguination, and their sera were stored at -70°C until use. A pooled sample prepared from the individual sera within an immunization group was applied to the antigen arrays immediately after thawing.

Fabrication of the Antigen Array—All the materials for antigen spots, if not stated otherwise, were obtained from Sigma. For the preparation of zymosan, a suspension of bakers' yeast cells was boiled for 1 h and then washed with physiological salt solution. This treatment exposes the cell wall component zymosan, which is particularly efficient in initiating the lectin and alternative pathways of complement activation. A hand-held spotting device, Microcaster (Whatman), was used for arraying the antigens according to the manufacturer's instructions. The spotting pins of our contact printing arrayer were ~ 500 μm in diameter. Antigens were spotted onto

homemade nitrocellulose-covered slides or Fastslides (Whatman) and stored at 4°C in a sealed bag until use or for a maximum of 4 weeks. On each array were printed three to four replicate features of 1:5 serial dilutions of each antigen. The starting concentration of the antigens was 1 mg/ml in PBS containing 0.05% sodium azide.

Hybridization Conditions for Complement Activation—Dried arrays were rinsed for 15 min in PBS just before use. Arrays were then incubated with sera in a humidified chamber at 37°C for 60 min. Serum complement activation took place with the array covered by a glass slide, which was kept at 1-mm distance from the array by plastic spacers. The amount of mouse serum required for one measurement was 600 μ l or less depending on the size of the array. The reaction was terminated by washing the array with PBS. Alexa Fluor 647-conjugated detecting antibodies goat anti-mouse IgG and anti-mouse IgM were from Molecular Probes, whereas the fluorescein-conjugated goat F(ab')₂ fragment to mouse C3 was obtained from MP Biomedicals. This polyclonal antibody reacts with all the fragmented forms of C3 including C3b, iC3g, and C3d. The detecting antibody or the mixture of the two antibodies diluted 1:5,000 in 5% skimmed milk powder in PBS was added to the arrays, which were then incubated with gentle agitation for 30 min at room temperature in the dark. The arrays were scanned after three more 10-min washes in PBS.

Analysis of Hybridized Microarrays—In a preliminary experiment, after preparing the array for scanning as described above, digital images of individual features of the array were taken on an Olympus IX-70 inverted fluorescence microscope equipped with a DP-50 digital camera using appropriate settings for fluorescein detection. Scale was determined by using a Bürker counting chamber as standard.

Slides were scanned on a Typhoon Trio+ imager (Amersham Biosciences) following standard protocols. Laser intensity was set to provide optimal signal intensity with minimal background and without saturated pixels. Data were analyzed with ImageQuantTL (Amersham Biosciences) software. Signal intensities were calculated by subtracting background from medians of signal intensity in a spreadsheet program (Microsoft Excel). For interassay comparisons fluorescence intensities were adjusted to give comparable readings for the highest dilutions of protein LA (pLA).¹ Overlays of false color images were prepared by Adobe Photoshop software.

RESULTS

Overview of the Antibody Array—We spotted various kinds of purified or complex antigens, proteins, nucleic acids, whole mouse serum, and particulate antigens, and a goat anti-mouse C3 capture antibody on nitrocellulose-covered glass slides. Two of the antigens were potent activators of the classical pathway: a fusion protein from bacterial Ig-binding proteins (protein LA) and heat-aggregated human immunoglobulins. Zymosan, on the other hand, was used as a known activator of the lectin and the alternative pathways. Bovine and human albumins, keyhole limpet hemocyanin (KLH), lysozyme, dinitrophenol-BSA conjugates, influenza virions, and zymosan were used as foreign antigens, whereas murine albumin and DNA were used as self structures. Arrays were incubated in mouse sera at 37°C to allow complement activation to take place. The generated C3b molecules form part of the C3 convertase complexes and permit the production of

¹ The abbreviations used are; pLA, protein LA; dsDNA, double-stranded DNA; JHD, J segment of heavy chain deleted; KLH, keyhole limpet hemocyanin.

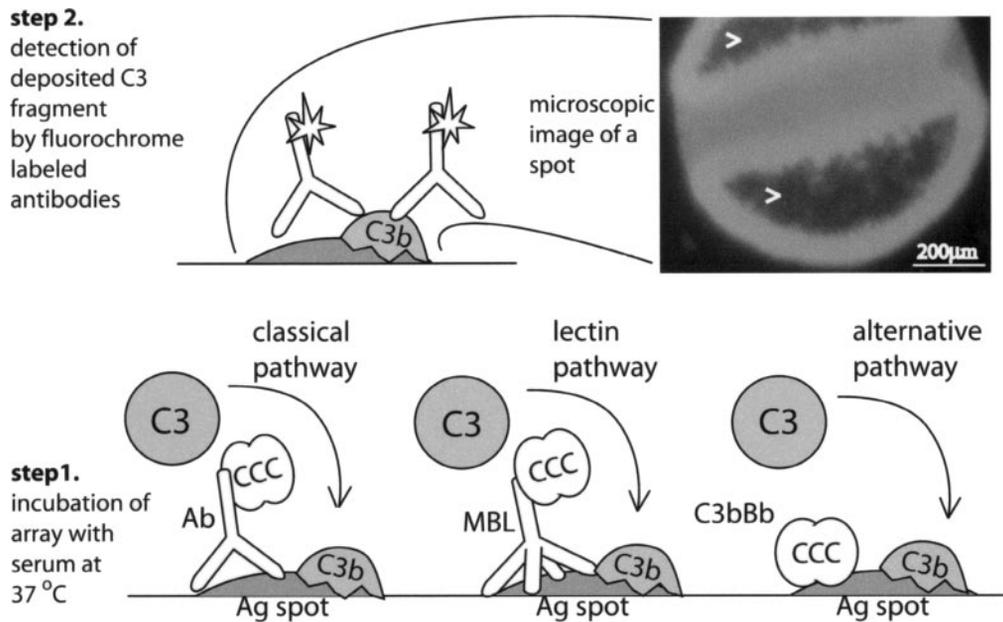


FIG. 1. **Schematic illustration and microscopic image of the C3 deposition antigen array.** Complement activation in the tested serum sample can be initiated by the binding of antibody (Ab) and C1q (classical pathway), lectins (e.g. mannose binding lectin (MBL)), or the alternative pathway C3 convertase complex (C3bBb) to antigen spots (Ag spot) of the array. As a result, a C3 convertase complex (CCC) forms on the features and generates reactive C3 fragments (C3b) that immediately form covalent bonds with nearby NH_2 and OH groups, including water molecules (Step 1). The alternative pathway convertase C3bBb forms directly on the activating surface. After washing out unbound C3, labeled antibodies are used for identifying features with deposited C3 fragments (Step 2). *Inset*, fluorescent microscopic image of a spot with deposited C3. Fine features imprinted by the spotting pin are readily distinguishable, confirming that practically there is no diffusion of the generated reactive C3b and fluorescence is strictly restricted to the area of printed antigen. *Arrowheads* mark shadows left by the split printing pin.

more C3b fragments. Covalent C3 fragment binding to the relevant array features was detected by fluorescently labeled antibodies as outlined in Fig. 1.

The first question to be answered was whether the short half-life of the reactive C3b component really restricts C3 deposition to the respective activating features of the array. We used a nitrocellulose-based array because earlier Western blot experience suggested that nitrocellulose was inert and suitable for incubation in fresh serum without nonspecific complement activation (23, 24). To confirm that the generated soluble C3b fragments are bound only in the area of the printed antigens we microscopically examined protein LA spots. It is evident in Fig. 1 (*inset*) that C3 signals had a sharp, high contrast border with no diffusion-derived transition of fluorescence between the spot and the background. As a result of the contact printing method the fine features (two crescent-like shadows) of the split printing pin clearly showed up in the image. This result suggests that diffusion of the generated soluble, reactive C3b is indeed a negligible factor on a micrometer scale, and therefore the measurement of complement C3 deposition can be applied to microarrays.

Activation of Complement and C3 Deposition on the Array—In addition to the antigens we included a dilution series of polyclonal C3-specific capture antibodies as a way to confirm the presence and compare concentrations of C3 in the serum samples. To exclude the possibility that C3 binds to

the features of the array by passive adsorption we added EDTA to the serum before incubation. EDTA chelates Ca^{2+} and Mg^{2+} ions, which are indispensable for complement activation. Addition of EDTA completely prevented C3 deposition on pLA, human IgG, and zymosan; the only remaining C3 signal came from the C3 capture spots and the spotted sera containing C3 (Fig. 2). To further confirm the specificity of the system we incubated arrays with serum from genetically C3-deficient mice (22) with the result that even the capture spots became negative. The arraying and hybridization conditions were therefore suitable for the specific detection of complement activation.

Sensitivity and C3 Dependence of the Method—Sensitivity of the method, in terms of the minimal amount of C3 detectable, was established by spotting serial dilutions of C3-sufficient and -deficient serum. There was practically no background signal from the serum of C3-deficient animals (Fig. 3A). Assuming serum C3 concentration of normal mice as 0.5 mg/ml and an average printing volume of 50 nl, 200 pg of C3 was still detectable on the array. Relative fluorescence intensity values in the range of $0\text{--}3 \times 10^7$ corresponded to $0\text{--}25$ ng/spot calculated C3 mass (Fig. 3B). Correlation coefficient of intra-assay spot-to-spot variation was less than 10% for spots containing 1 mg/ml antigen but increased at lower antigen concentrations. To assess the dependence of the method on C3 concentration we reconstituted sera of genet-

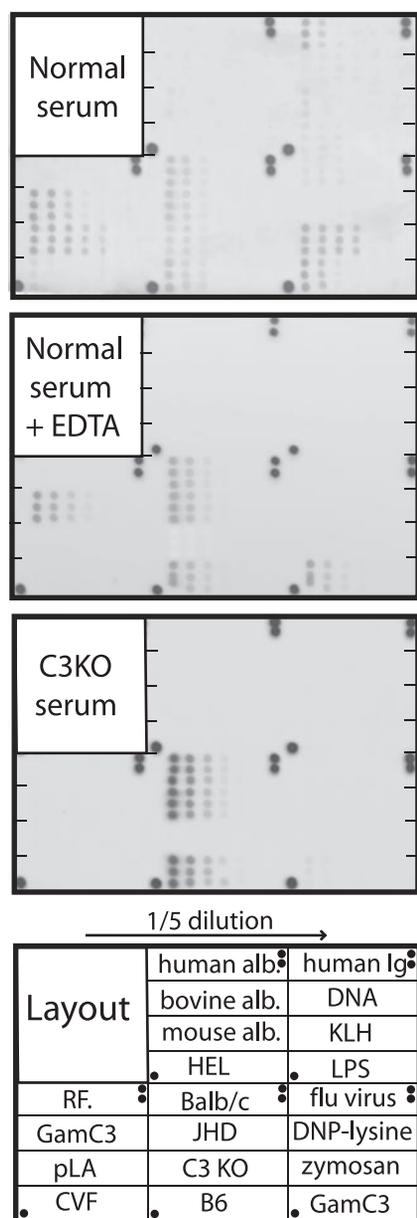


FIG. 2. Complement deposition requires activation of the complement system. A medium density antigen array was prepared with the components indicated in *Layout* using contact printing on a nitrocellulose surface. Orientation spots mark subarrays in the *lower left* and *upper right* corners. All antigens were spotted in six different dilutions and three parallels. The arrays were incubated in normal serum, normal serum supplemented with 20 mM EDTA, or C3-deficient serum for 1 h at 37 °C. Pictures show scanned images after labeling C3 with FITC-conjugated F(ab')₂ antibody fragments. *RF.*, rheumatoid factor; *GamC3*, goat anti-mouse C3; *CVF*, cobra venom factor; *alb.*, albumin; *HEL*, hen egg lysozyme; *LPS*, lipopolysaccharide; *flu*, influenza; *DNP*, dinitrophenol. Serum samples from BALB/c, JHD, C3KO, and C57/B6 mouse strains were also printed as shown.

ically C3-deficient mice with different amounts of C3-sufficient normal serum. The assay proved to work even after a hundredfold reduction in C3 concentration, although it became less sensitive in the case of zymosan-induced C3 dep-

osition (Fig. 3C). It is important to note that zymosan can initiate both the lectin and alternative pathways. This two-component activation might be responsible for the observed break of the curve showing epitope density-dependent complement activating efficiency.

Two-color Microarrays for Simultaneous Detection of Antibody and C3—Individuality in serum complement immunoprofiles is expected mostly from differences in antibody repertoires. Therefore the measurement of C3 deposition is particularly interesting in combination with antibody binding measurements. To assess the possibility of concurrent determination of bound antibodies and C3 fragments we analyzed sera from mice immunized with BSA. Detection antibodies against C3 were FITC-labeled, whereas those against IgG were Alexa Fluor 647-conjugated. Whereas no C3 signal was detectable on the albumin spot after reacting with nonimmune mice serum (Fig. 2), bound C3 and IgG were readily detectable on BSA when the array was incubated in immune serum (Fig. 4). Moreover cross-reactivity against human serum albumin was also observed. Interestingly some C3 deposition was also observed on the murine albumin spots as well. No C3 deposition was detected on an unrelated protein, hen egg lysozyme. Zymosan on yeast particles is a well known activator of the lectin and alternative pathways of complement, and as Fig. 4 shows C3 deposition was indeed induced without the binding of any detectable IgG.

Characterizing Qualitative Differences of the Humoral Immune Response—The nature of antigen-specific antibodies that are generated following immunological challenge depends on the nature of the challenge itself. Thus, strong inflammatory signals promote the appearance of antibodies with the ability to sustain inflammation partly by fixing complement. To assess the potency of the C3 deposition array in discriminating the subtleties of a humoral immune response, we compared C3 deposition on KLH from sera of non-immune animals, animals immunized with protein only, and animals immunized with protein in complete Freund's adjuvant. Serum from naïve animals showed no C3 deposition on KLH, whereas significant amounts of C3 were detected, along with Ig, when sera of immunized animals were tested (Fig. 5, A and B). Of note, the difference in C3 deposition was more pronounced than in Ig binding between serum samples from animals immunized with or without complete Freund's adjuvant. The pattern of C3 deposition and IgG binding to pLA was not different in the three groups (Fig. 5, C and D).

The ability to activate the complement system influences the pathogenicity of autoantibodies. To address the use of the C3 deposition array in a murine model of systemic lupus erythematosus, we measured antibody binding and C3 fragment deposition on dsDNA spots in sera from normal and severely ill MRL/lpr mice. As expected, serum from the autoimmune animals contained antibodies against dsDNA. These antibodies activated the complement system as indicated by the deposition of C3 fragments (Fig. 5E).

FIG. 3. Sensitivity of detection and influence of C3 levels in the serum. Dilutions of C3-sufficient (normal, \times) and -deficient (C3KO, \blacksquare) serum samples were spotted and developed by the C3-specific FITC conjugate (A and B). Relative fluorescence intensities were obtained from the scanned images, confirming the absence of signals from C3-deficient mouse serum and showing the sensitivity of C3 detection (B). C3-deficient serum (\blacksquare) was reconstituted with C3-sufficient serum (\times) to give 90% (\bullet) and 1% (\triangle) relative C3 content. Antigen arrays were incubated in these samples, and relative fluorescence intensities indicating C3 deposition on the two model antigens pLA and zymosan were obtained (C and D). Note that only the latter two diagrams show the result of C3 deposition by complement activation as opposed to printed C3 on the first two diagrams.

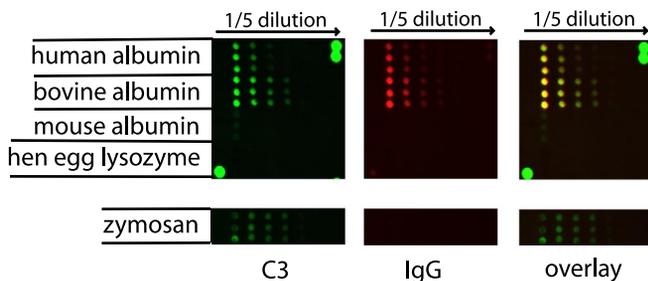
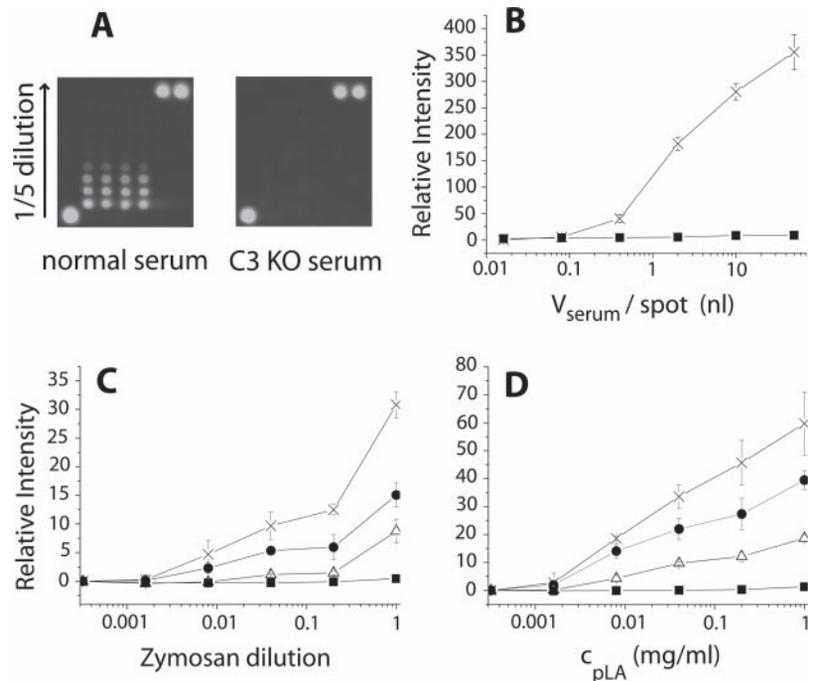


FIG. 4. Simultaneous detection of C3 and IgG binding. An antigen array was incubated in anti-BSA immune serum, and then deposited C3 and bound IgG were detected simultaneously with FITC- and Alexa Fluor 647-conjugated antibodies, respectively. Zymosan activated complement without antibody binding. Images are false color images of the two scans and their overlay.

DISCUSSION

A proteomics approach for characterizing the reactivity of the immune system requires identification of the repertoire of molecules that can be specifically recognized by the “detection units” of the system. The best known such unit is the antibody molecule, which can be tailored to recognize practically any structure depending on the need of the organism. Antibodies are not the only soluble detection units, however, and the mere presence of an antibody that binds a particular antigen does not necessarily have medical relevance. A further layer of humoral immune recognition is provided by the complement system. Importantly sites of complement activation are physically marked by covalently bound C3 fragments, a situation ideal for applying to solid-phase immunoassays, including protein microarrays. Antigens spotted on an array can activate the complement system by any one of the three described pathways, and the generated reactive C3 frag-

ments will react with any neighboring molecule (including the spotted antigen and the bound antibodies) in a temporally, and therefore also spatially, confined manner. Thus, complement activation will result in the deposition of C3 only to the antigen spots that initiated the activation, marking the antigen spot clearly for identification (Fig. 1).

In this study we show for the first time that the measurement of complement activation on an antigen array is easily achievable and provides important information beyond antibody binding. Using substances that efficiently trigger complement activation in an antibody-dependent (pLA) or -independent (zymosan) way, we demonstrated that high signal to noise ratios were achievable (Fig. 3, C and D), confirming that complement activation was indeed restricted to the activating spot. Inert proteins, such as albumin or lysozyme, did not give any signal in normal sera, supporting the specificity of the technique. Note, however, that KLH, a relatively huge aggregated protein of 10^5 – 10^7 daltons, induced C3 deposition even in the sera of nonimmune animals (Figs. 2 and 5). The most likely cause for this activation is the presence of complement-activating natural antibodies against KLH in mouse serum as it has been described in humans (25). The binding of these antibodies is probably below the detection limit, similar to the undetectable binding of BSA-specific antibodies to murine serum albumin in Fig. 4 where C3 deposition was observed without IgG signal.

In the presence of the chelator molecule EDTA, which blocks all three pathways of complement activation, no C3 deposition was observed (Fig. 2). Thus, C3 was not passively adsorbed but was deposited by covalent attachment exclusively as the result of an active complement pathway. In addition to its covalent linkage to the array features, detecting C3 fragments as a readout of complement activation is also

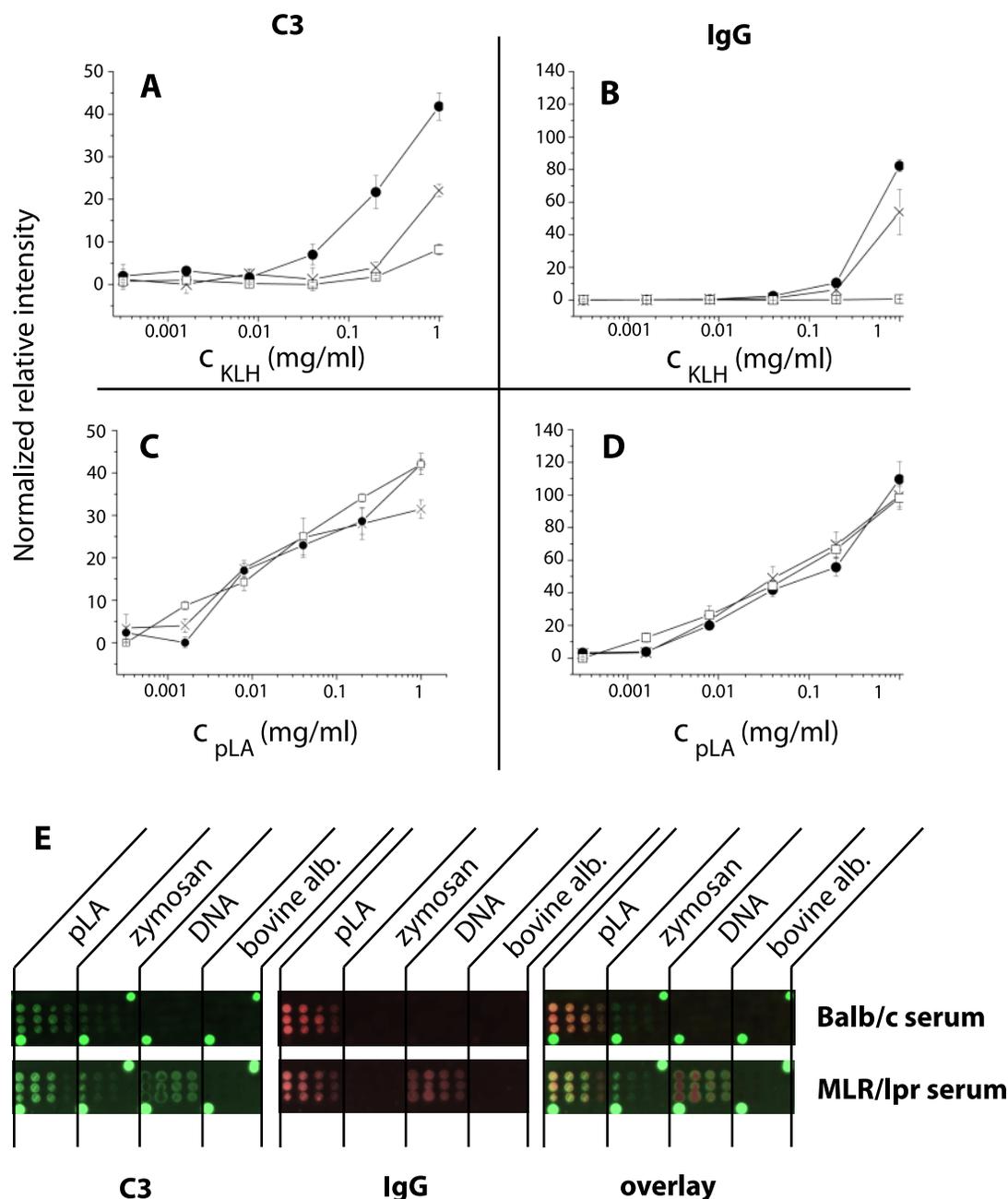


FIG. 5. **Qualitative differences in serum antibody response as reflected by C3 deposition.** A–D, antigen arrays were incubated in sera from three different groups of mice: nonimmune (□), KLH-immunized (×), and KLH + complete Freund’s adjuvant-immunized (●) animals. Diagrams show fluorescence intensities of deposited C3 (A and C) or bound IgG (B and D) on KLH (A and B) or pLA (C and D) spots. E, the ability of autoantibodies against dsDNA to activate complement was tested by incubating the arrays in serum from normal or MRL/lpr mice. Shown are false color images of the fluorescein and Alexa Fluor 647 scans and their overlay. *GamC3*, goat anti-mouse C3; *alb.*, albumin; *mIgG*, mouse IgG.

advantageous because of the presence of an amplification loop (19): a single C3 convertase can generate several more convertases, which in turn cleave more and more C3 molecules. The process is comparable to the tyramide signal amplification method (26) that is used for increasing the sensitivity of immunoassays and *in situ* hybridizations.

In view of the fact that healthy individuals possess func-

tionally equivalent amounts of complement proteins, most of the individual variability is expected to originate from antibody-dependent complement activation. In our experiments, as expected, complement activating properties of the antibodies were influenced by the immunization protocol: C3 deposition was more pronounced in the serum of animals immunized with inflammation-inducing adjuvants (Fig. 5). This result is in

agreement with the known complement activating properties of the antibody isotypes produced during inflammatory conditions. In addition to epitope density and affinity of the interaction, it is the isotype (27–29) and the glycosylation status (30) of the antigen-bound antibody that determines whether complement is activated. Antibody isotypes with complement activating properties are more effective in clearing viral infections like human immunodeficiency virus (31) and more efficient in inducing self-damage (32, 33). Furthermore the relative abundance of antibodies against the same target but with distinct isotypes may determine the development of autoimmune disease (34). Simultaneous detection of more than two or three antibody isotypes is not generally achievable due to limitations in multicolor detection. Thus, contemporaneous measurement of relative contribution to antigen binding by e.g. IgM, IgG1, IgG2, IgG3, IgG4, and IgA is not yet feasible. Measurement of complement activating properties of bound antibodies can be a simple and meaningful alternative to solve these problems. Additionally because the test is carried out in undiluted sera, the assay more closely mimics events in the body unlike immunoassays where dilutions in reaction buffers potentially mask protein interactions.

We used dsDNA as a representative autoantigen to assess the usefulness of the C3 deposition array in an autoimmune disease model. The presence of antibodies against dsDNA is a diagnostic criterion for systemic lupus erythematosus (35), and their isotype and affinity, factors influencing complement activation, have been reported to correlate with disease severity (36, 37). Accordingly an early study showed that titers of complement-fixing dsDNA antibodies correlated with disease activity and renal damage (38). Serum from severely ill animals indeed showed both antibody binding and C3 deposition onto dsDNA in our assay (Fig. 5). The special ringlike C3 signals obtained with higher concentrations of dsDNA may have resulted from the sensitivity of complement activation to epitope density. We did not observe similar C3 signals in the case of protein antigens, so it is more likely to be a peculiar trait of dsDNA-antibody interactions.

Multiplex systems for autoantibody profiling are promising new tools for the diagnosis and classification of autoimmune diseases (13, 39) with the potential of guiding the development of treatment strategies (40). Taking into account that complement plays an important role in a range of autoimmune (33, 41, 42), inflammatory (43), ischemia-reperfusion-mediated (44), and degenerative diseases (45), we propose that the simultaneous detection of antibody binding and complement deposition in a high density antigen array format may become a clinically useful method with diagnostic and prognostic values.

The disadvantage of the complement deposition array is the need for serum samples with an intact complement system. To prevent degradation of complement proteins sera need special handling: immediate use after the drawing of blood or storage at -70°C . As an alternative solution, when only classical pathway-mediated activation is studied, antibody bind-

ing and complement activation can be separated in time: the tested sample can be heat-inactivated, and antibody-depleted serum can be used as a complement source in two sequential incubations. Another potential drawback is that because the test is carried out with undiluted sera larger volumes might be required for measurement as compared with an ELISA test. However, as the volume of serum required for a test is still below 0.6 ml, this feature cannot be considered a real disadvantage for human applications.

CONCLUDING REMARKS

Currently there is no multiplex diagnostic method that allows the identification of complement-activating substances and antibody targets simultaneously. The technique we describe here, in addition to the potential clinical value of allowing the measurement of an essential function of antibodies in a microarray format, may also help in better understanding the interactions of the complement system on a proteomic scale.

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