Complementing antibody profiles: assessing antibody function on antigen microarrays

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Abstract

Antibody effector functions other than neutralization depend on interactions with soluble and cellular components of the immune system. Antigen recognition is usually oligoclonal, with the different clones of antibodies belonging to different classes, subclasses, glycoforms and having different affinities and epitope specificities. Thus, composition of immune complexes determines biological effects mainly via interactions with FcR and complement proteins. Antibodies are capable of triggering any of the three pathways of complement activation and antigen recognition of complex antigens often results in the activation of more than one pathway. These events can be tracked in a multiplex format using antigen microarrays, where complement products bind to elements of the microarray. By controlling cation concentrations and detecting various complement components (C1q, C4, C3) contribution of the different pathways can be identified. Parallel measurement of antibodies and complement proteins provides a novel way of looking at interactions between antigen and antibodies. We propose the use of immune complex signatures, composite depictions of antibody and complement content of immune complexes characterizing healthy and diseased populations. Normalized interquartile ranges of antibody binding (IgM, IgG) and complement deposition (C4, C3) are projected onto radar charts to produce patterns that can distinguish normal and altered immune responses.

The comprehensive interaction studies of serum antibodies and complement with arrays of antigens can generate profiles for a better understanding of disease mechanism.

1 **Antibodies, complement and arrayed antigen**

The complement system participates in wide ranging biological phenomena, starting from regulation of organogenesis and regeneration through mediation of inflammation to promoting opsonic phagocytosis to modulation of adaptive immunity. This variability is partly due to the fact that the system has about 30 components and also due to the presence of a variety of receptors on different cell types. While complement activation usually implies the triggering of one of the pathways which then proceeds to the formation of the membrane attack complex, it is important to note that the cascade of enzymatic cleavage events can be blocked at several points by natural inhibitory and regulatory molecules, producing a different outcome. Antibodies are usually associated with classical pathway, where antigen-bound IgM or IgG provides binding sites for C1q, leading to activation of the associated serine proteases C1r and C1s. However, it is important to note that not only classical but also the lectin and alternative pathways can be triggered by antibodies bound to their target [1]. Accordingly, IgG molecules have sites associated with the triggering of these pathways, as shown in Figure 1.

Antigens coupled to a solid surface can serve as targets of antibodies, as long as important conformational epitopes are not lost due to structural changes induced by adsorption, covalent linkage, dehydration or other technical factors associated with production. In our experience nitrocellulose is a universal surface that can bind all kinds of macromolecules, such as proteins, lipids, nucleic acids and their various complexes. Random adsorption to the nitrocellulose sponge allows access to any part of the antigen. While the use of peptides instead of whole proteins has certain advantages [2] in antibody profiling, only native antigens reflect in vivo immune complex formation. Multiple

epitopes recognized within the same antigen, determined by the diversity of the immune response, promote complement activation due to the increased density of antibodies. Otherwise, epitope density on the microarray can be controlled by adjusting concentration of the antigen solution used for array printing. At a given epitope density antibodies with higher affinity will be more potent complement activators as the time spent in antigen-bound form will be longer. Therefore, on part of the antigen, quality and epitope density, on part of the serum, antibody diversity and affinity is reflected by complement activation.

Human IgG subclasses differ greatly in their effector potential, both in FcγR and complement mediated events. Switching to these isotypes is determined by the nature of the antigen (and adjuvant), and while dominance of certain isotypes can characterize immunity, usually one can expect different IgG subclasses to be bound to arrayed antigens. The ratio of these subclasses can determine the extent of complement activation.

Another important property of IgG molecules is the composition of the N-linked carbohydrate moiety: different glycoforms possess different effector functions, again both by FcγR [3,4] and complement [5] mediated processes. Terminal fucose, mannose and N-acetyl glucosamine molecules present in certain glycoforms are preferred sites for MBL. Lectin pathway-initiated complement activation has been shown to contribute to antibody mediated effector functions in disease [6]. In summary, the nature and extent of complement activation induced by a certain antigen in a given serum sample integrates a wealth of information about the bound antibodies.

The pathway that leads to C3 deposition can be identified by looking at complement proteins on the antigen array under three different conditions: in the presence of both Ca^{2+} and Mg^{2+} ions, in the presence of Mg^{2+} ions with Ca^{2+} ions chelated by EGTA, and in the absence of cations, all being chelated by EDTA (Fig. 2). The presence of C1q indicates involvement of the classical pathway; its binding is not affected by cation concentrations. C4 deposition results either by classical or lectin pathway initiated events, both being dependent on Ca^{2+} ions; C4 in the absence of C1q therefore indicates lectin pathway activation. Finally, C3 is generated by all three pathways but only the alternative pathway is active in the absence of Ca^{2+} ions. Figure 2B demonstrates that a bacterial superantigen, fusion protein LA, triggers both the classical and the alternative pathway by capturing IgG from the tested serum.

2 Interpreting complement activation as a function of antibody binding

Allowing serum proteins to react with arrayed antigens under conditions that support complement activation, we have wide choice of serum proteins for measurement. As for immunoglobulins, except for IgD, all classes and subclasses have been shown to be detectable on antigen arrays [7]. Therefore the choice mainly depends on the purpose of the study; we have mostly used IgG detection for the assessment of vaccination effects and to monitor autoreactivity. Concerning complement proteins, we have successfully detected C1q, C1INH, C4, C3 and C5b-9 neoantigen. Since C3 plays a central role in complement activation and its density correlates with C5 convertase activity [8], its measurement provides a good overall picture about complement activation events. Though it is actually not C3 but its fragments (C3b, iC3b, C3dg) that are bound to the

array, from here on we neglect this fact and continue to call array-bound forms C3 in general. It is important to note that parallel detection of immunoglobulins and complement proteins has certain technical tricks, which were recently described [9]. While our group focuses on complement function, other approaches of interpreting combined immunological binding events are also being developed [10].

2.1 2D profiles – comparing antigens

The ability of antigens to capture immunoglobulins and complement proteins (Fig. 3A) spans a very wide range starting from non-detectable to very high values. Binding of immunoglobulins can be explained by the presence of natural antibodies and prior exposure to the antigen. This latter category, the "reactome", includes environmental antigens, accidental or intentional inoculation of pathogens and self antigens. Complement activation on the antigen is more difficult to predict, being influenced by non-immunoglobulin and immunoglobulin factors as well. The physico-chemical nature of the antigen determines the binding of recognition molecules such as pentraxins, lectins, and of complement regulatory proteins such as factor H or C4BP. These molecules can up- or downregulate the complement cascade, influencing the amount of different complement products generated. Thus, the ratio of proteins along the C4-C3-C5b-9 axis can vary as a function of these proteins, irrespective of the bound immunoglobulins. Complement activating ability of immunoglobulins was discussed in the previous section.

The ideal concentration of antigens for arraying has been shown to be between 0.1-1.0 mg/ml. In order to compare two different antigens on a molar basis, equimolar solutions should be printed on the antigen arrays. While this is usually not the case, the use of

different dilutions allows a rough qualitative comparison to be made, especially for protein antigens that have similar molecular weights. Figure 3B shows the relative composition of immune complexes formed on 5 different antigens. This kind of comparison is very precise, as protein interactions take place on the very same array under identical conditions. On the other hand, special reagents may be required to confirm that identical – ideally known – amounts of the antigens are present on the array surface. This is achieved by the use of peptide tags and tag specific antibodies in the case of recombinant antigens [11].

2.2 2D profiles – comparing serum samples

When the comparison of reactivity of different serum samples is the goal, reactions take place in different reaction chambers (Fig. 3A). This necessitates the use of quality control and normalization procedures that compensate either for technical or for biological variation. On the technical side, array printing can introduce variability in the amount of antigen printed and in antigen quality. On the biological side, absolute concentrations of immunoglobulins and complement proteins and functional activity of complement system shows individual variability, due to genetic and environmental factors. The measurement of these biological variations is often not the purpose of antigen array experiments, rather these factors should be compensated for. As an example, normalization for total immunoglobulin M levels has been proposed as a means to render IgM binding results comparable [12]. Serum concentrations of complement C3 and C4 vary in the range of 1.0–1.85 g/l and 0.2–0.45 g/l, respectively; in a similar manner, normal complement activity, as assessed by CH50 measurement, also spans a $\log_2$ range. Our approach is to determine the relative complement activating properties of antigen-bound antibodies,

therefore we normalize results to complement deposition on obligate complement activating substances [9]. This approach allowed the distinction between antibody profiles induced by different adjuvants [13] and to monitor the progressive qualitative change of antinuclear antibodies in a murine model of lupus erythematosus [14].

2.3 2D profiles – plotting antigens and serum profiles

Alterations in the reactivity pattern of serum antibodies reflect changes in the immune system. Antibody profiling has been used to characterize these changes both with the purpose of identifying markers of disease and of generating multiparameter algorhytms for defining disease status. Visualizing these changes in two dimensions can provide an interesting and informative functional view of humoral immunity; an example is shown in figure 3B.

3 Description of immune complex content on a population level: immune complex signatures

Experimental data, in agreement with the expectable correlations [15] suggested that it is mostly IgM and IgG that determines complement depositions to self antigens. Detection of these two classes of immunoglobulins seems inevitable in order to interpret effector functions. On the complement side, alternative pathway and its amplification loop were identified as important contributors to disease pathogenesis. Thus, the measurement of both C4 and C3 is required to assess relative contribution of alternative and the other two pathways. Current microarray scanning technology allows the simultaneous detection at four different wavelengths, so technically concurrent measurement of IgM, IgG, C4 and C3 is feasible. Another concern could be the interference of the detecting secondary

antibodies with each other and thereby the skewing of signals by more abundant proteins. Our pilot experiments indicated that this was not the case (data not published) so we set out to measure the four proteins in parallel.

Measurement of the binding of four different proteins to an antigen raises the issue of how to present data in a way that is both simple and informative. Radar charts – also called cobweb charts or star plots - are suitable for displaying multivariate data in two dimensions. They consist of a sequence of spokes or axes that start from the same point and represent one variable each. Data points belonging to one observation are plotted on each axis and are then connected by a line. The resulting star-like plot can be used for the qualitative characterization of an observation. Typically many observations are plotted on a single chart so that observations with similar or dissimilar star shapes can be visualized. We adapted this method for displaying data ranges. Observations are interquartile ranges of values expressed as percentage of maximal observed values. Observations for individual samples are star shaped lines, whereas observations for populations are star shaped areas. These areas can be displayed on separate charts or as an overlay (fig 4). IgM and IgG are displayed on opposite axes to represent natural, low affinity responses in contrast to acquired, high affinity secondary responses. In a similar manner, C3 and C4 are displayed on opposite axes, accounting for early and later events during complement activation. In the resulting plot the IgM-C4 region represents classical pathway/natural immunity while the IgG-C3 region stands for induced immunity with AP activation.

4 Perspectives

From the technological point of view, multiplex measurements are becoming common practice both in research and in *in vitro* diagnostics. Detecting antibodies with dozens or more specificities with simultaneous isotype determinations are possible now using planar or bead arrays. Understanding the relationship between antibody binding patterns and biological effects in healthy and ill populations will require the standardized collection of data and the generation of databases on one hand. On the other hand, incorporation of the next step of events following antibody binding, such as complement activation or FcγR triggering, into these datasets, could further illuminate individual variability and promote personalized medical interventions.

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Conflict of interest

J.P., Z.S. and K.P. are partly emloyed by Diagnosticum Inc., the company that licences rights on the pending patent „Measurement of complement activation on antigen arrays" from Eötvös Loránd University and the Hungarian Academy of Sciences.

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Figure legends

Figure 1. Interactions of an IgG molecule and their effects on complement activation

A polyclonal or oligoclonal response resulting in better epitope coverage on the target antigen will improve complement activation by providing C1q binding sites in close proximity. Epitope density on the antigen or the antigen microarray also determines the proximity of IgG molecules, and thereby C activation. IgG can serve as an acceptor for the covalent binding of C3b, promoting the generation of alternative pathway (AP) C3 and C5 convertases. The C1 complex binds to different Ig isotypes with different efficiencies (IgG3>IgG1>>IgG2>>IgG4), explaining the variable classical pathway (CP) initiated C fixing properties of IgG subclasses. Terminal sugar moieties of the N-linked carbohydrate group determine MBL binding and lectin pathway (LP) activation.

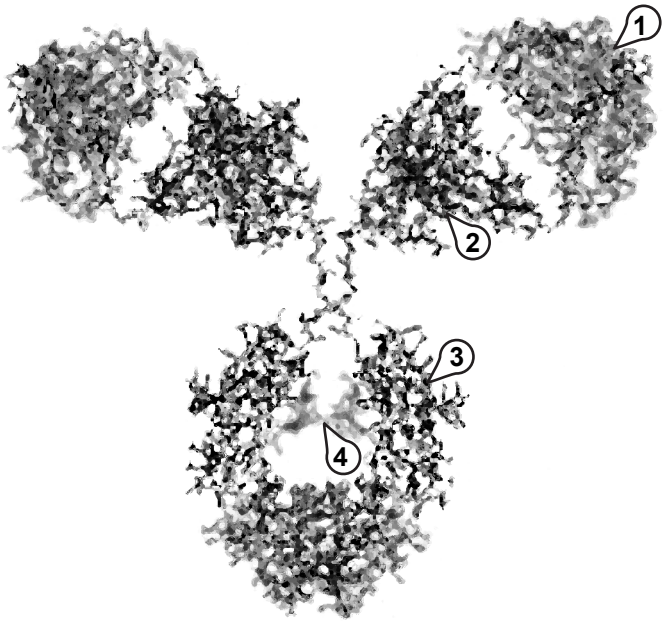
Figure 2. Scheme for complement pathway identification

A, Matrix for identifying single pathways: by measuring the binding of three complement proteins (C1q, C4, C3) and using three different reaction conditions with respect to availability of bivalent cations, pathways contributing to C3 deposition on a particular feature of the array can be identified.

B, Examples: combined activation of classical and alternative pathway by bacterial superantigen fusion protein pLA; alternative pathway activation by yeast extract; C3 capture by antibodies as positive control.

Figure 3. 2D immune profiles: interpretation of C3 fixation as a function of Ig binding.
A, On a given array different antigens appear at different positions depending on the ratio of IgG and C3, reflecting properties of both the antigen and immunity. The same antigen appears at a different position when comparing immune reactivities of different serum samples. B, The two approaches can be combined, giving rise to complex views of disease associated humoral immunity.

Figure 4. Immune complex signatures for characterizing immune complex composition in populations.
A, In order to compare the binding of different proteins to antigen arrays, a relative scale is used. Boxes represent interquartile ranges, whiskers stand for minimum and maximum values. B, To visualize 4 parameters of immune complex composition (IgM, IgG, C3, C4) a radar chart with four axes is used with the relative percentage scale. C and D, Interquartile ranges (from part a) of a given population (group 1, C; group 2, D) are projected onto the radar chart, resulting in patterns characterizing the given population. E, Areas representing different populations can be overlaid for comparison.



site	binding partner	IgG variants	role
1	epitope	idiotype	determines Ag binding
2	C3b		AP promotion
3	C1q	isotype	CP initiation
4	MBL	glycoform	LP initiation

Figure 1

A

Classical

	Ca Mg	- Mg	- -
C1q	+	+	+
C4	+	-	-
C3	+	-	-

Lectin

	Ca Mg	- Mg	- -
C1q	-	-	-
C4	+	-	-
C3	+	-	-

Alternative

	Ca Mg	- Mg	- -
C1q	-	-	-
C4	-	-	-
C3	+	+	-

Protein LA

zymosan

anti-human C3

B

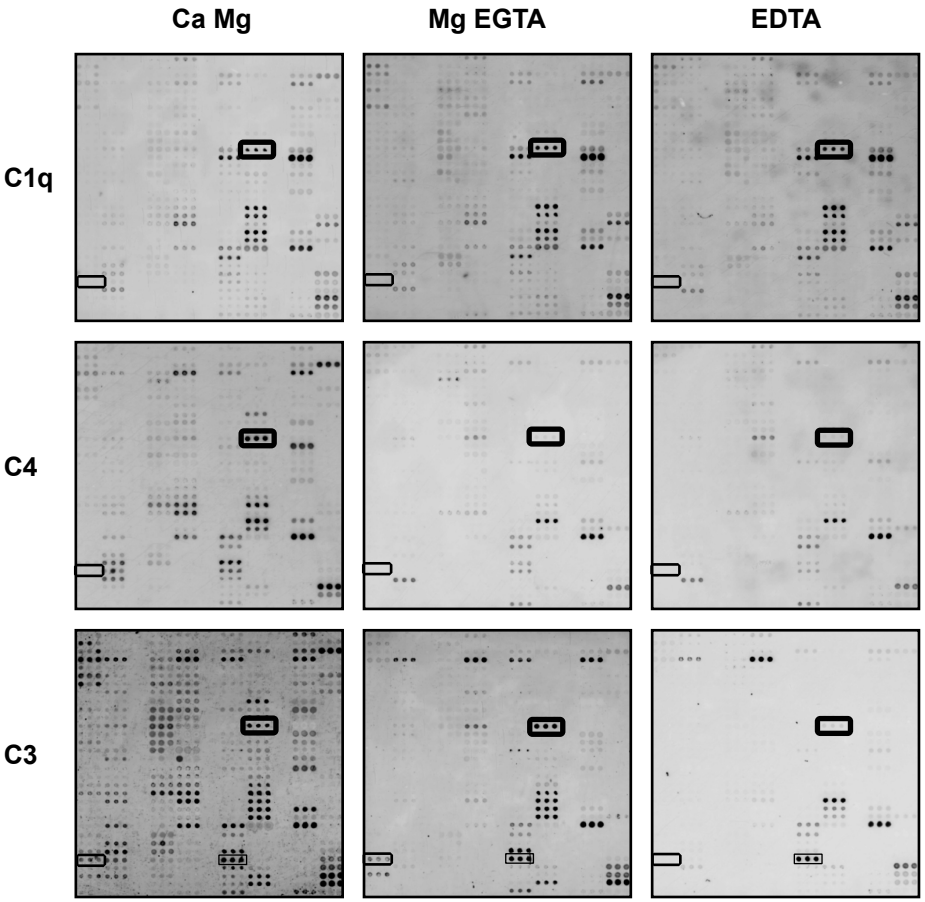


Figure 2

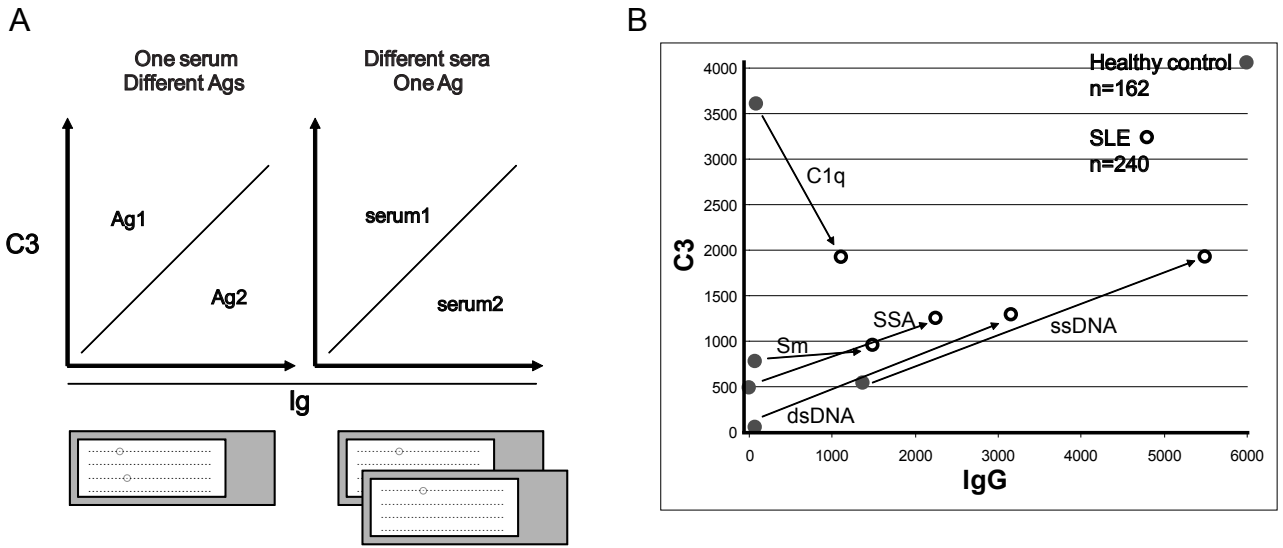

A
One serum
Different Ags
Different sera
One Ag
C3
Ag1
Ag2
serum1
serum2
Ig
B
Healthy control
n=162
SLE
n=240
C1q
SSA
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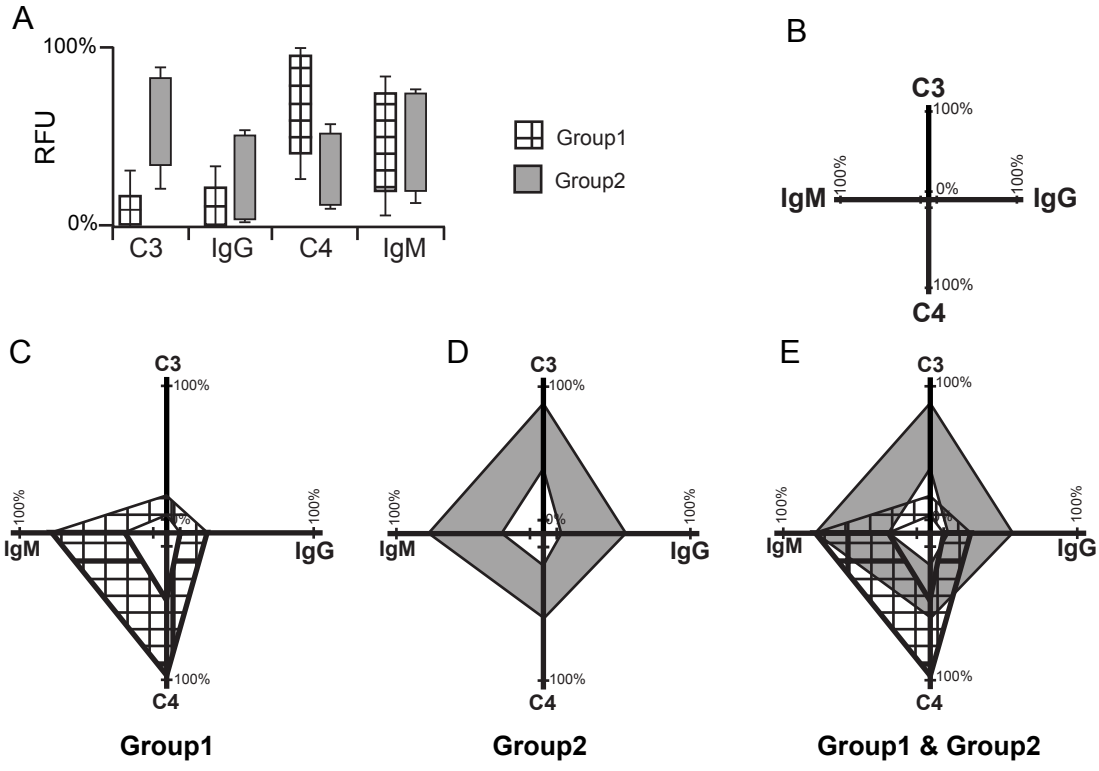
Figure 3


A
100%
RFU
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C3
IgG
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Figure 4