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RAPID COMMUNICATION

Autoantigen arrays for multiplex analysis of antibody isotypes

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We describe here a microarray-based method for multiplexed, antigen-specific assessment of immunoglobulin (Ig) subclasses. We used 1152-feature arrays composed of 140 antigens or antigen fragments to detect isotype-specific mAb, to quantitatively monitor changes in isotype mAb concentration, and to profile antigen-specific antibody isotype production in a murine model of autoimmunity. This platform can be easily adapted to a variety of applications, and has the potential to elucidate mechanisms that govern development and evolution of antibody responses in *in vivo* and *in vitro* systems.

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Antibodies of the IgG isotype can be further divided into subclasses, which differ in their ability to carry out specific effector functions (*e.g.* binding to complement or Fc receptors). In mice, switching to IgG2a is preferentially induced by the prototypical T helper 1 (Th1) cytokine interferon-gamma (IFN-γ), while IgG1 switching is predominately driven by interleukin-4 (IL-4), a Th2-associated cytokine [1, 2]. Importantly, Th1 and Th2 cytokines appear to counter-regulate each other both *in vitro* and *in vivo* [3], a phenomenon that is particularly relevant in the context of human and animal models of disease. For example, induction of Th2 responses inhibits experimental autoimmune encephalomyelitis, a Th1-mediated animal model of human multiple sclerosis (MS) [4, 5]. Overactive Th2 responses are associated with atopic processes [6], suggesting that the Th1/Th2 balance determines the development of autoimmune or allergic conditions [7]. Hence, the evaluation of antibody isotypes can provide substantial insight into immune system regulation and disease pathogenesis.

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Abbreviations: Ig, immunoglobulin; **Th**, T helper

The most common methods for the characterization of antibody isotypes (Western blotting and ELISA) tend to be laborious and offer limited multiplexing capacity. Recently, autoantigen arrays have proven useful in the study of autoantibody responses in numerous models of autoimmune disease, including MS and systemic lupus erythematosus (SLE) [8–12]. To evaluate the utility of this technology in the detection of antibody subclasses, we used a robotic arrayer to spot various connective tissue disease-associated autoantigens on glass slides. These autoantigens included native proteins, recombinant proteins with hexahistidine (His)-tags, and several overlapping peptides. (See Supplementary Tables 1 and 2 for a complete list of antigens and peptides.) The slides were probed individually with IgG1 or IgG2a anti-His mAb, followed by either Cy5-conjugated goat anti-mouse IgG1 antisera or Cy3-conjugated rat anti-mouse IgG2a mAb (Fig. 1a). This approach permitted isotype-specific detection of anti-His mAb to epitope-tagged antigens (Fig. 1b). These reactions were highly specific, as signal was only detected when secondary antibody was matched with the anti-His mAb of the appropriate isotype (data not shown). In addition, incubation of arrays with secondary antibodies alone produced no signal (data not shown). This technique proved suitable for applications beyond detection of antibodies directed against epitope tags. We detected the interaction of IgG1 (9A9) and IgG2a (2.73) mAb with their cognate antigens, U1-small nuclear ribonucleoprotein/Smith

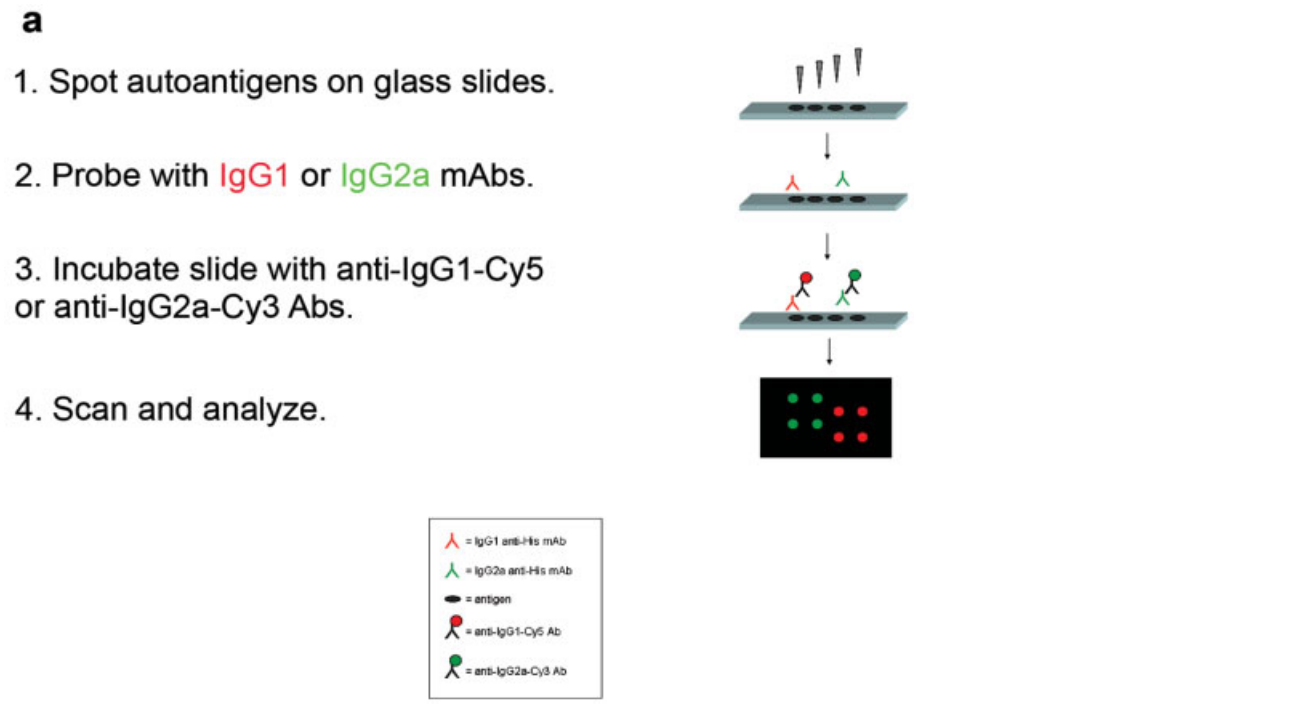
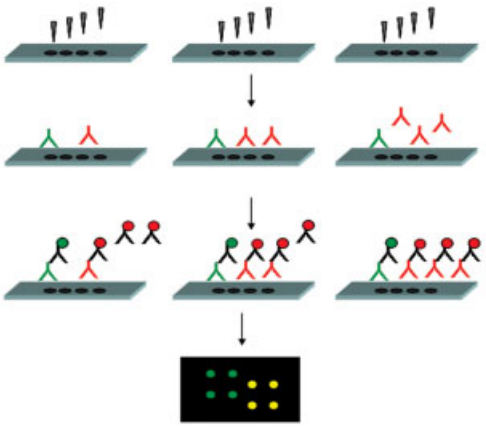


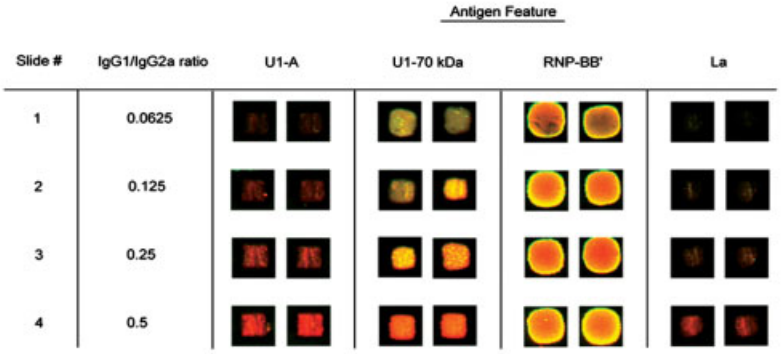
Figure 1. Use of autoantigen microarrays to detect isotype-specific mAb. (a) Diagram outlining the experimental scheme. (b) His-tagged antigens were spotted on arrays, and the arrays were subsequently incubated with IgG1 anti-His (top panel) or IgG2a anti-His mAb (bottom panel), followed by the appropriate fluorophore-conjugated isotype-specific secondary antibodies. U1-A, U1-C, U1–70 kDa, RNP-BB', Ro52, La, hnRNP-B1, hnRNP-D, beta-2 glycoprotein-1, and anti-mouse IgG antigen features are highlighted by the white boxes.

a

1. Spot 6X-his-tagged autoantigens on glass slides.
2. Probe with IgG1 and IgG2a anti-6X-his mAbs. Keep IgG2a Ab concentration constant, while varying IgG1 concentration on consecutive slides.
3. Incubate slides with anti-IgG1-Cy5 and anti-IgG2a-Cy3 Abs.
4. Scan and analyze.



b

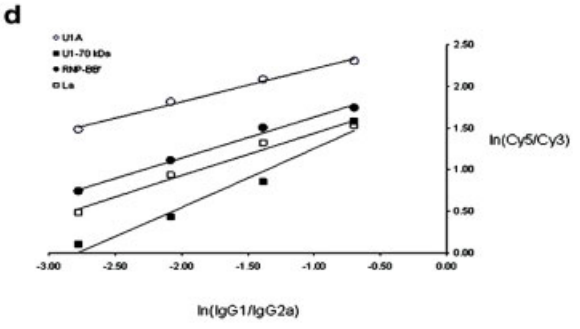


c

Cy5/Cy3 ratio

Slide #	IgG1/IgG2a	U1-A	U1-70 kDa	RNP-BB'	La
1	0.0625	4.38	1.10	2.09	1.81
2	0.125	6.13	1.54	3.04	2.54
3	0.25	8.02	2.33	4.46	3.73
4	0.5	10.01	4.86	5.70	4.61

d



Antigen	Equation (y = mx+b)	R^2
U1-A	y = 0.40x + 2.61	0.991
U1-70 kDa	y = 0.70x + 1.96	0.965
RNP-BB'	y = 0.49x + 2.12	0.991
La	y = 0.51x + 1.95	0.976

Figure 2. Use of microarrays to determine relative levels of IgG1 and IgG2a mAb. (a) Diagram outlining the experimental scheme. (b) Consecutive arrays (four total) were probed with varying concentrations and ratios of IgG1 and IgG2a anti-His mAb. Select antigen features from individual arrays are shown. (c) Quantitation of fluorescence intensities for selected features. Numbers represent the median Cy5/Cy3 ratio for individual antigen features (eight features per antigen) from each slide. (d) The data from (c) were plotted, and best-fit curves were drawn for each antigen. The equation of the line for each antigen is given in the table below the graph. For each antigen, the correlation coefficient value (R^2) was very close to 1.0, indicating that the Cy5/Cy3 ratio was nearly linear over the range of IgG1/IgG2a ratios tested.

(U1-snRNP/Sm)-A (U1-A)/U2-B" and U1–70 kDa, respectively (Supplementary Fig. 1). We also identified a U1–70 kDa-derived peptide corresponding to amino acids 219–238 that is recognized by the 2.73 mAb. This region (in the carboxyl terminus of U1–70 kDa) is rich in arginine/aspartic acid repeats, and has previously been shown to contain the 2.73 epitope [13]. These results demonstrate that autoantigen array technology may be useful for performing fine epitope mapping of autoantibody responses [8].

To determine whether autoantigen arrays can be used to monitor changes in antibody levels, we utilized the scheme diagrammed in Fig. 2a. Consecutive arrays were probed with IgG1 anti-His mAb, varying concentration from 6.67 μg/mL to 53.33 μg/mL. The arrays were simultaneously incubated with IgG2a anti-His mAb, which was kept constant at a concentration of 3.33 μg/mL (yielding IgG1/IgG2a ratios of 0.5, 0.25, 0.125, and 0.0625, respectively). After washing, the arrays were incubated with an antibody solution containing anti-IgG2a-Cy3 and anti-IgG1-Cy5 antibodies. The slides were then scanned, and the Cy5/Cy3 ratio – a direct indicator of the relative IgG1/IgG2a ratio – for individual antigen features was calculated. For the four His-tagged autoantigens analyzed – U1-A, U1–70 kDa, RNP-BB', and La – the Cy5/Cy3 ratio was linear over a nearly one-log concentration range (Figs. 2b–d), demonstrating that autoantigen arrays are capable of detecting subtle changes in antibody concentration.

Microarray technology has also proven useful in the quantitation of antibody subclasses in biological samples, such as serum, cerebrospinal fluid, peritoneal fluid, and synovial fluid [8]. We examined whether autoantigen arrays could be used to quantitate specific antibody isotypes in serum derived from mice primed with the mineral oil pristane. BALB/c mice given a single intraperitoneal injection of pristane develop a lupus-like condition, marked by renal pathology and the production of autoantibodies directed against several lupus-associated autoantigens [14]. Arrays were incubated with BALB/c mouse serum obtained 12 months after pristane treatment, followed by Cy5-conjugated goat anti-mouse IgG1 antisera or Cy3-conjugated rat anti-mouse IgG2a mAb. Adapting the scheme outlined above (Fig. 2a), we also quantified relative levels of IgG1 and IgG2a antibodies in sera derived from pristane-primed mice. The autoantigen microarray technique permitted simultaneous detection and quantitation of serum IgG1 and IgG2a antibodies directed against the U1-A, RNP-BB', and U1–70 kDa components of the U1-snRNP/Sm complex. Furthermore, IgG1/IgG2a values for most antigens were validated by ELISA (data not shown). Interestingly, within a given pristane-primed mouse, the IgG1/IgG2a ratios varied considerably among these three autoantigens (Supplementary Fig. 2). These data suggest that autoantibody responses to specific antigens are regulated in an independent fashion. Thus, assessment of total IgG1 and IgG2a levels in serum may not accurately portray immune deviation at the level of individual antigens, a previously reported feature of the pristane-induced model of SLE [15]. Moreover, multiplexed, antigen-specific antibody isotyping studies may provide insight into local cytokine environments that govern class switching during interactions between autoreactive T cells and autoreactive B cells.

Recently, other investigators have applied protein arrays to the study of autoantibody profiles in SLE. Mohan and colleagues constructed protein arrays consisting of approximately 30 known glomerular and glomerular basement membrane antigens. These studies identified distinct patterns of IgG and IgM autoreactivity to select glomerular antigens in human lupus patients. While some of the reactivity clusters correlated with disease activity, other autoantibody clusters did not [10]. Importantly, Th1-associated autoantibodies (predominantly of the IgG2a isotype) have been proposed to play a major role in the pathogenesis of murine lupus [16–18]. Thus, the use of isotype-specific secondary antibodies may reveal isotype-dependent autoantibody signatures.

In summary, our results demonstrate the utility of autoantigen microarrays in the characterization of autoantibody profiles and multiplexed antigen-specific isotype analysis. Importantly, the autoantigen array and ELISA techniques may differ in their ability to detect antibodies of varying affinities. However, a major advantage of the array technique is that it can be applied to the analysis of multiple antibody isotypes (to potentially thousands of antigens) within a given sample on a single glass slide. Conversely, approaching such questions by ELISA requires the separate incubation of distinct, antigen-specific secondary antibodies with antigens in multiple wells. Moreover, with suitable reagents and equipment (*e.g.* a multi-color scanner), the array technique can be adapted to analyze three or more antibody isotypes simultaneously. Such studies have the potential to uncover isotype-dependent autoantibody profiles, and may provide novel insights into autoantibody associations and mechanisms of disease progression.

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