The application of next generation sequencing to the understanding of antibody repertoires

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In the decade since the human genome sequence was declared complete, the development of next generation sequencing (NGS) or “deep” sequencing to deliver cost-effective genomic sequencing has influenced advances beyond its primary application and changed the research landscape in many other areas. This review will survey recent applications of NGS which have broadened the understanding of natural antibody repertoires (the “antibodyome”) and how these evolve in response to viral infection. We will also report examples where deep sequencing of binding populations, derived from both natural and synthetic repertoires, have been used to benefit antibody engineering. This knowledge will ultimately lead to the design of more effective biological drugs and vaccines.

Keywords: next generation sequencing, antibody, repertoire, antibodyome, humoral immune response, synthetic library, deep sequencing

INTRODUCTION

Since the human genome was declared complete in April 2003, the costs of genomic sequencing have been reduced by orders of magnitude and available tools exist for data analysis, so that access to massively parallel sequencing is no longer the exclusive realm of large genome centers. Instead, next generation sequencing (NGS) technologies such as 454 pyrosequencing and Illumina Solexa clonal bridge amplification methods have become widespread and can be operated by individual investigators, allowing the scope of their use to be expanded (1–7). In this review, we will focus attention toward how NGS can reveal the sequence space occupied by antibodies in their recognition of antigens. Specifically, we will concentrate on how NGS informs our knowledge of natural antibody repertoires and how the humoral immune system adapts to infection, how it can be used to improve the efficiency of screening and how it can be applied to the design of new systems for the discovery of novel biologics.

THE DEVELOPMENT OF NATURAL ANTIBODY REPERTOIRES

The sequencing of natural antibody or immunoglobulin (Ig) repertoires poses a different challenge to genomic sequencing, as the antibodyome (“the knowledge of the complete set of antibodies in an individual”) is in constant flux whilst the genome is relatively invariant. The diversity of the antibodyome in adult humans is estimated from the numbers of antibody expressing B cells within the body which is approximately $10^{10}$–$10^{11}$. However, this population changes quickly with time and tissue distribution, resulting from daily turnover and replenishment of B cells, which potentially introduces new sequences (8). This sequence multiplicity is due to several natural mechanisms, the most important being somatic recombination and rearrangement of two or three sets of gene segments into a single unique gene. Each antibody is made of the products of two genes, encoding heavy and light chains, which provide further intricacy. In the case of the heavy chain gene, at least 56 variable (V), 23 diversity (D), and 6 joining (J) segments exist that are rearranged by RAG1 (recombination activating gene 1) and RAG2 recombinases in a process known as V(D)J recombination that brings VDJ segments together (light chain genes lack D regions). Imprecise fusion at the junctions from insertions and deletions through the activity of terminal deoxyribonucleotidyl transferase creates additional combinatorial diversity (junctional diversity) and the sequence from the V-D, through the D segment to the D-J junction (or V-J junction in light chains) is known as the complementarity determining region (CDR) 3 that is critical for the antibody’s antigen specificity (9, 10).

Therefore, most of the effort to understand human antibody repertoire has focused on deep sequencing of the CDR3 regions, especially the heavy chain CDR-H3, to understand repertoire diversity. The uniqueness of individual’s antibodyome was highlighted by results from sequencing these regions from two human subjects, which discerned a slight bias in the pairing of D and J but not in V-DJ. Also, between two individuals, there were CDR-H3s in common although this was a small fraction of the CDR-H3 diversity, estimated to be between 3 and $9 \times 10^9$ (10). Sequencing efforts have demonstrated that certain VH segments are over-represented in the natural human repertoire but it was uncertain whether this was determined by genetic, disease, age-related, or environmental factors (11, 12). However, a study on two monozygotic twin pairs demonstrated that whilst the variation in the naïve VH and DJ segment use is strongly dictated by the individual’s germline genetic background, even in the case where one twin was affected by multiple sclerosis (MS), the CDR-H3 repertoires were highly specific to the individual. The authors suggested that even with common gene segment profiles, there is likely to be a different antibody response to common environmental exposure (13).

Interestingly, in contrast to the CDR-H3 repertoire, recent investigation in the rearrangement in the light chain CDR-L3 found that a surprisingly large proportion of CDR-L3 (more than 20%) was shared across individuals, which indicates that an
intrinsically, an immune response is triggered by B cells that produce antibodies against specific antigens. These antibodies are crucial for neutralizing viral infection and preventing disease spread. As a result, it is essential to understand the factors that influence antibody response and selection in HIV-1 infection.
antibodies grouping into 30 clonotypes likely to have been derived from different progenitor B cells. The repertoire was dominated by two to three VH families and a subpopulation containing a large number of mutations. The VH response was consistent with the abundances observed for the VH repertoire highlighting the importance of the cellular repertoire in determining humoral immunity. Seven days post-immunization, 16 out of 34 of the identified CDR-H3s in the serum repertoire map exclusively to sequences found in the PBC database, and may be derived from recently activated plasma blasts in transit to the bone marrow, whereas the remainder were likely to be expressed from plasma cells that had migrated to the bone marrow. There was also evidence of oxidative modification from mass spectrometry but it was not known if this was an in vivo post-translational modification contributing to additional diversity in the antibody libraries (26). In another analysis of B cell distribution, the repertoire was compared on both sides of the blood-brain barrier (BBB) in MS and patients suffering other neurological diseases. In some patients, common VH sequences were identified on both sides of the BBB, but the data indicated that only a few B cells migrate through the BBB are retained in the central nervous system (CNS). In MS patients the IGHV4 segment predominates, suggesting the framework to be particularly suited against MS antigens (27).

**OVERCOMING THE LIMITATIONS OF NGS IN ANTIBODY REPertoire ANALYSIS AND ITS UTILITY IN SCREENING**

As highlighted in the examples above, a deficiency of NGS is that it is not currently possible to sequence both of the two chains of the antibody in a single read. Therefore, when using the common methods, no information on the natural VH:VL pairing, which is crucial to discern native antibodies, can be obtained beyond inference from frequency analysis of sequencing separately both variable domains. However, despite this limitation, 21/27 scFvs constructed from pairing together the most abundant VH and VL genes from immunized mice were expressed in E. coli and bound antigen with nanomolar affinity. Yet, pairing differentially ranked heavy and light variable domains in a full antibody format yielded a subnanomolar IgG in HEK 293F cells (28). Indeed, the method of repertoire mining of VH and VL abundances through NGS of splenocytes, isolated from immunized mice, was compared with a phage panning approach of the same cDNA. While both methods provided antibodies with comparable affinities, clones identified by repertoire mining showed higher selectivity for the antigen. Antibodies selected by phage display were barely detected by NGS, and conversely, mining the V repertoire identified antigen-specific antibodies that were not selected by phage display (29). This study demonstrated the expression bias of traditional phage display methods and the complementarity of using both approaches to isolate both rare and abundant binding sequences, thus supporting results from an earlier study by Ravn et al. (30). Here, NGS data were used to retrieve scFvs that could bind to the target with high affinity without the need for primary screening. Indeed the methods enabled the retention of clones that could have been lost during screening in small-scale soluble expression formats. A similar method has been used for screening an antigen in a more complex environment, where the antigen was not purified but displayed on the bacterial surface. NGS analysis of a scFv library that bound to bacterial cells expressing the target, versus a control population, provided information necessary to synthesize scFv binders to IL-6 (31).

A proteomics approach that combines high resolution LC-MS/MS analysis of purified and digested fragments of serum antibodies referenced against databases derived from the NGS reads of the B cell repertoire has been developed to provide more precise information for VH:VL pairing (32). However, this is a difficult problem to solve using proteomics because VH and VL abundances do not correlate, due to an excess of VL secreted into the serum, and the fact that VL sequences have lower complexity which results in VL sequences sharing partial identity (26).

Recently, an elegant solution to this problem has been described, which isolated more than \(5 \times 10^6\) single B cells individually in the microwells of a high-density microplate (33). Poly-dT beads were added to the wells and, after cell-lysis, the mRNA was captured on the beads and emulsified for cDNA synthesis. The VH:VL pairs were linked by PCR and sequenced by paired-end long reads using Illumina technology. This experiment was performed on repertoires post-immunization for antigen-specific plasmablasts against tetanus toxoid and for memory B cells after influenza vaccination. Some of the VH:VL pairs identified were expressed in IgG format and they all demonstrated affinities in the subnanomolar-nanomolar range.

**APPLICATION OF NGS TO FUTURE ANTIBODY ENGINEERING**

Antibody display libraries derived from human PBMCs or hybridomas immortalized from B cell populations have been successfully used in recent decades to isolate binders against a wide range of targets, despite a lack of detailed knowledge of the repertoires (34, 35). With the advent of NGS, analysis of the natural naïve repertoires from which libraries have been constructed has become possible.

In an early paper, the diversity of a phage displayed combinatorial library generated from the IgM repertoire of 654 healthy human donors was precisely quantified by deep sequencing pre- and post-selection using long-read pyrosequencing (12). Variable domain PCR amplicon and rolling circle amplified shotgun methods allowed an efficient assessment of diversity, as well as the correct assignment of heavy and light chain pairing. A novel application of Hidden Markov Model (HMM) accurately identified CDR regions. The sequencing results revealed that all germline families were present and a high degree of somatic mutations in CDR1 and CDR2 provided additional complexity to a library that was estimated to be similar in diversity to the number of transformants (\(3.5 \times 10^{10}\)). The library was subjected to panning against 16 targets and pairing preferences were observed for heavy and light chains. This information was used to produce combinatorial libraries that mimic the natural repertoire both in length and sequence diversity. A synthetic Fab library was fabricated in which all six CDRs are diversified by synthetic enzymatic codon addition method allowing precise control of amino acid additions at each position in the CDRs, to recapitulate those found in nature. The library was subjected to panning against a diverse panel of receptors, growth factors, antigens, enzymes, and peptides. Binders
were isolated for all antigens with nanomolar affinities measured for 6 out of 10 antigens [36].

Larman et al. [37] describe an interesting synthetic approach where CDR sequences were designed using a HMM model of “contact” and “non-contact” states for amino acid positions based upon known antibody-antigen complexes. These sequences were synthesized in a releasable format on a DNA microarray, assembled into a single framework scFv library, panned by ribosome display against poliovirus receptor-related 4 (PVR-L4) and the binding output submitted to NGS analysis. Of the top 25 most abundant clones post-selection, four were found to specifically bind human mammary epithelial cell (HMEC)-expressed PVR-L4 by FACS-staining analysis.

Further scrutiny of natural systems, the use of modern synthetic approaches, surveying the enrichment process, and examining the resulting targeted antibody repertoire will better inform the design of next generation synthetic libraries [38–40]. This will undoubtedly improve the performance of synthetic libraries, many of which have been poor in functionality due to degenerate designs that do not respect loop length diversity, amino acid, or structural preferences of natural systems.

CONCLUSION

Major challenges still remain in the use of NGS for antibody research with respect to reliably identifying the heavy and light chain pairs, as well as the bioinformatic analysis of the output. However, in a relatively short time span, NGS has impacted heavily on our understanding of the mechanism of the humoral response to viral insult, antibody clonal selection, and the chemical and structural nature of the binding landscape of the variable domains. Yet, whilst it has enabled researchers to take small steps forward in the quest for deriving the ultimate binders from their systems, NGS methods also highlight the need to learn more from both natural and synthetic repertoires. Sequencing combined with proteomics techniques will provide better resolution of the natural systems, and modern library synthesis methodologies will allow greater use of rational and combinatorial approaches. These will permit scientists to create designer libraries that will specifically address certain classes of antigens or determine greater biophysical stability, manufacturability, longer shelf-life, and improved pharmacokinetic and pharmaco-dynamics properties than existing antibody therapeutics.

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